Outpatient antibiotic use in acute respiratory infections in Ho Chi Minh City, Vietnam

Thesis

OUTPATIENT ANTIBIOTIC USE IN ACUTE RESPIRATORY INFECTIONS IN HO CHI MINH CITY, VIETNAM

by

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Abstract

Acute respiratory infections (ARI) are among the most frequent infectious diseases in children worldwide, particularly in developing countries. Antibiotics are very often prescribed or purchased without prescription for the treatment of ARI, although viruses are recognised as the predominant pathogens [1-7].

The aim of the work presented in this thesis was to quantify antibiotic use in outpatients with ARI in Children's Hospital 1, Ho Chi Minh City, Vietnam, to identify the viral and bacterial respiratory aetiologic agents and to assess the impact of antibiotic use on the selection of resistant bacteria in the intestinal flora.

Two prospective descriptive studies were conducted in the outpatient clinic: one in ARI patients and the other in healthy children. The epidemiology, presentation and treatment characteristics of children with ARI in the outpatient clinic were described. Antibiotics were prescribed in 99.6% of 563 patients while respiratory viruses were detected in 72.5% among these patients with the use of multiplex PCR in respiratory specimens. Antibiotic use was considered inappropriate in 67.7% of cases, according to evidence-based guidelines and detected pathogens. Besides antibiotics, other treatments such as oral bronchodilators, oral corticosteroids, antihistamines, and mucolytic agents were commonly used at the rates of 57.6%, 10.3%, 11% and 11%, respectively, and in most of the cases, were not in accordance with the current guidelines. We observed a short-term selection of resistant *Enterobacteriaceae* in patients' intestinal flora resistant not only to the antibiotic class the patients received but also co-selection of resistance to other rarely used antibiotics. HPLC assays were developed with high sensitivity and specificity to determine the presence of 6 beta-

lactam antibiotics in the urine. Antibiotic use before presentation as determined by HPLC (32%) was significantly higher than that reported by parent interviews (21%). Antibiotic use in Vietnam is largely unrestricted leading to overuse and overprescription for uncomplicated ARI.

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Declaration

Other than the assistance indicated in the acknowledgements, the work presented in this thesis is my own work and has not been submitted for a degree or other qualifications to any universities.

Abbreviations

Acute respiratory infections	ARI
Adenovirus	AdV
Bordetella parapertussis	BPp
Bordetella pertussis	BPt
Children's Hospital 1	CH1
Chlamydophila pneumoniae	CPn
Chlamydophila psitacci	CPs
Crossing point value	Cp value
C-reactive protein	CRP
Deoxyribonucleic acid	DNA
Escherichia coli	*E.coli*
Extended spectrum beta - lactamase	ESBL
Haemophilus influenzae	Hin
High Perfomance Liquid Chromatography	HPLC
Human Bocavirus	BoV
Human Coronavirus	CoV
Human Enterovirus	EV
Human Metapneumovirus	MPV
Human parainfluenzaviruse 1	PIV-1
Human parainfluenzaviruse 2	PIV-2
Human parainfluenzaviruse 3	PIV-3
Human parainfluenzaviruse 4	PIV-4

Human Parechovirus	PeV
Human Rhinovirus	hRV
Influenza virus A	Flu A
Influenza virus B	Flu B
Integrated management of childhood illness	IMCI
Legionella pneumophila	LP
Lower respiratory infections	LRI
MacConkey	MC
Mycoplasma pneumoniae	MP
Nasopharyngeal aspirate	NPA
Nose and throat swab	NTS
Polymerase Chain Reaction	PCR
Real Time	RT
Respiratory Syncytial Virus A and B	RSV A/B
Ribonucleic acid	RNA
Solid phase extraction	SPE
Streptococcus pneumoniae	SP
Upper respiratory infections	URI
World Health Organisation	WHO

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Chapter 1

Introduction

1.1 Acute respiratory infections in children

1.1.1 Overview of acute respiratory infection

1.1.1.1 Acute respiratory infection in children: definitions

Acute respiratory infections (ARI) are defined as acute infections of the airway and classified into acute upper respiratory infections (URI), which affect the nose, middle ear, and pharynx, and acute lower respiratory infections (LRI), which refer to infections of the epiglottis, trachea, bronchi, bronchioles and lungs [8]. URI, which are seen mostly in outpatients, are more widespread and important in terms of numbers, but clinically usually less severe and only seldom lead to hospitalization or mortality. However, URI are a source of significant morbidity leading to a remarkable socioeconomic burden [9].

ARI constitute one of the leading causes of childhood morbidity and mortality in developing countries. Most children have on average four to six ARI every year [8]. Most of these are limited to the upper airways and self-limiting, but a small percentage can progress to lower respiratory tract infections such as bronchiolitis and pneumonia. Globally, there are about 156 million new cases of clinical pneumonia in children aged less than 5 years annually of which 151 million episodes are in the developing world. The incidence of clinical pneumonia is highest in South-East Asia (0.36 episodes per child-year), followed by Africa (0.33 episodes per child-year) and the Eastern Mediterranean (0.28 episodes per child-year). According to the World

Health Organization (WHO), childhood pneumonia is the leading cause of mortality and is responsible for about 19% of all deaths in children aged less than 5 years [10]. In 2000, approximately 1.9 million (95% CI 1.6-2.2 million) children died from ARI throughout the world, 70% of them in Africa and Southeast Asia [11].

1.1.1.2 ARI symptoms, signs and classifications

There is a wide variety of symptoms and signs of ARI including sore throat, cough, runny nose, stuffed nose, difficulty breathing, and fever or ear problems. For clinical management, WHO has divided ARI into four classifications: very severe disease, severe pneumonia, pneumonia and no pneumonia (cough or cold). A child with very severe disease is a child who has any danger sign such as "not able to drink or breastfeed", "convulsions", "unconsciousness", "vomiting everything" or "severe malnutrition". The second degree of severity is severe pneumonia in which the child has chest indrawing or stridor, while a child who has fast breathing without chest indrawing and stridor is classified as having "pneumonia". Fast breathing is defined by respiratory cut-off rates developed by WHO (50 breaths per minute for 2-12 month infants and 40 breaths/minute for 1-5 year old children). Chest indrawing is defined as inward movement of the bony structure of the chest wall with inspiration. The last and mildest classification is "no pneumonia (cough or cold)" in which the child has no danger signs, no chest indrawing and no fast breathing [8, 12].

Table 1-1 Classification of ARI

Classification of ARI	Symptoms and findings
Severe disease	Unable to drink or breastfeed, OR Unconscious, OR Convulsion, OR Vomiting everything
Severe pneumonia	Chest indrawing, OR Stridor in calm child
Pneumonia	Fast breathing
No pneumonia (cough or cold)	No signs of pneumonia or severe disease

1.1.1.3 Epidemiology of ARI

ARI occurs year-round but there are fluctuations in the occurrence of different respiratory pathogens worldwide, with geographic and seasonal variations. In temperate climates, significant seasonal variations are seen in the incidence of viral respiratory tract infections, with peaks during the cold months (winter). This is explained by the fact that cooling of the nasal airways results in an inhibition of respiratory defences and an increased susceptibility to infection. Another possible explanation is that crowding indoors in the winter could increase the exposure to viral respiratory pathogens [13, 14]. In tropical regions, where the average temperature is higher and there are less fluctuations in temperature, variations in incidence are still seen though less clearly: viruses may circulate throughout the year and peaks may concur with either lower temperatures or increased rainfall. An increased incidence in the rainy season is attributed to a similar mechanism in which people spend more time

indoors and hence have increased human contact, including with people with viral respiratory infections [15].

Respiratory syncytial virus (RSV) has clear and characteristic seasonal epidemiology and in tropical climates usually coincides with rainfall during the rainy seasons as has been described in Vietnam [16], Malaysia [17], Singapore [18], India [19], Papua New Guinea [20], North-East Brazil [21], Kenya and the Gambia [22, 23]. In temperate countries, a very clear seasonal variation in the prevalence of RSV infections is seen with peaks occurring during the cold, winter months. In temperate climates, influenza epidemics occur predominantly during the winter months [24] while in tropical regions such as Singapore[18], India, North-East Brazil and Senegal [25], influenza virus infection peaks during the months with the highest rainfall and humidity. Human Metapneumovirus (hMPV) and Parainfluenza virus-3 (PIV-3) frequently coincide with the RSV season, while for Parainfluenza virus-1 (PIV-1) and Parainfluenza virus-2 (PIV-2), biannual outbreaks have been described.

1.1.2 **Aetiology of ARI**

The majority of ARI are caused by viruses and most of these are mild, self-limited illnesses and mostly involve only the upper respiratory tract. Bacterial respiratory infections are less common but may result in more severe or fatal disease. The estimated case-fatality rate for bacterial pneumonia due to *Streptococcus pneumoniae* (SP) and *Haemophilus influenzae* (Hin) in developing countries is more than 50 times higher than the case-fatality rate for infection due to RSV or parainfluenza viruses [26].

1.1.2.1 Viral agents

Viruses are found as the causative agent in 35-87% of children with acute respiratory infections depending on diagnostic techniques [1, 2, 4, 7, 22, 27-31]

The most frequently found viral pathogens in ARI include RSV, PIV-1, 2, 3, and 4, influenza virus A (Flu A) and influenza virus B (Flu B), human rhinoviruses (hRV), human coronaviruses (CoV) and adenoviruses (AdV). The prevalence of detected respiratory pathogens depends on different geographic areas, study settings, age of study participants as well as severity of ARI (URI or LRI). RSV is described as the most common agent in most studies worldwide [1, 2, 16, 19, 21, 31-34], whereas, in certain areas, other viruses were found as the most predominant respiratory virus. In Fortaleza, Brazil, among 175 children less than 5 years of age with ARI, hRV was most identified, at 45.6% [35]. In Madagascar, a prospective study of ARI in children between 2 to 59 months in the community hospital showed that hRV was the dominant detected respiratory virus, at 20.5% [36]. In Taiwan, enterovirus (EV) outnumbered other viral respiratory viruses detected among hospitalised and outpatient children in 1998 and 1999 due to outbreaks of EV 71 [7]. Among hospitalised patients, particularly in children under 2, RSV was identified as the most prevalent virus [7, 16, 21, 31, 32, 34] while hRV was the most frequently detected virus in the outpatient group, according to studies in Brazil and Madagascar [35, 36]. In northern Taiwan, AdV and EV were identified throughout the year as the two most common viruses in paediatric outpatients with acute, febrile URI [4].

Mixed viral and bacterial infections occur frequently at rates ranging from 0.3% to 22% [1, 2, 4, 27, 29, 33, 37].

Several novel viral pathogens associated with ARI have been recently described in the literature, including novel CoV (CoV-NL63, HKU1) [38, 39], hMPV [40], human Bocavirus (BoV) [41] and novel polyomavirus (WU and KI). For the latter two, the association with disease has not been fully established.

Recent studies on the prevalence of viral and atypical bacterial pathogens in children with ARI in developing countries are summarised in the Table 1-2 below:

Table 1-2 Studies of viral and atypical bacterial aetiology of ARI in children using multiplex PCR

Country	India [19]	Jordan [32]	Hong Kong [42]	Nepal [34]	Brazil [21]	Vietnam [16]	Kenya [31]
Year Published	2007	2008	2009	2009	2011	2011	2010
Age (years)	<5	<5	<5	<3	<5	<13	<12
Total Number patients in study	301	326	475	629	407	309	759
In-patient (%)	45%	100%	100%	100%	52%	100%	100%
Co-infection Rate	7	25	4	1	40	20	7
RSV	20	43	8	14	37	24	34
hRV	-	11	4	-	19	4	-
AdV	-	37	5	-	25	5	4
Flu A & B	3	1	11	7	3	17	6
PIV	16	0	9	10	8	7	8
CoV	-	1	4	-	3	8	10
MPV	4	3	1.5	1	10	7	3
BoV	-	18	-	-	19	16	2
MP	-	0	2	-	10	-	-
CPn	-	5	0	-	1	-	-
PCR negative	65	22	53	70	15	28	44

FluA: Influenza virus A; FluB: Influenza virus B; PIV: Human parainfluenza viruses; RSV: Respiratory Syncytial Virus; hRV: Human Rhinovirus; CoV: Human Coronavirus; BoV: Human Bocavirus; MPV: Human Metapneumovirus; AdV: Adenovirus; MP: *Mycoplasma pneumoniae;* CPn: *Chlamydophila pneumoniae.*

1.1.2.1.1 **DNA viruses**

1.1.2.1.1.1 Adenoviruses (AdV)

Respiratory infections caused by AdV are frequent during childhood. Detection rates of AdV in children with ARI varied from 4% to 37% in different studies [16, 21, 31, 32, 42]. However, most cases tend to be self-limited and induce serotype-specific immunity [43]. Prolonged asymptomatic carriage of AdV (up to 2 years in some cases) may occur in the tonsils of children. The clinical significance of AdV isolated from the throats of children must therefore be interpreted with caution.

1.1.2.1.1.2 Human Bocavirus (BoV)

BoV belongs to the *Parvoviridae* family but there is no relation between BoV and other respiratory pathogens. Although first discovered in 2005 from nasopharyngeal aspirates in children with respiratory tract infections [41], a causative relation between BoV and disease is not fully established. BoV is commonly detected together with other pathogenic respiratory viruses. BoV may lead to asymptomatic persistence or prolonged viral shedding, similar to other human parvoviruses that have the capacity for asymptomatic persistence. BoV could be either reactivated or cause transient co-detection triggered by (lytic) replication of another pathogen.

Detection rates of BoV varied from 2% to almost 20% in respiratory samples of children with ARI in developing nations [16, 21, 31, 32, 42]. Several studies have found a statistical association between BoV and acute respiratory symptoms. Viral DNA has been detected in blood of children with a respiratory infection, in faecal samples of diarrhoeal patients with or without respiratory symptoms and seroconversion has been shown to some degree. However, most studies have

limitations and the criteria to prove a causal relationship have not been adequately met [44].

1.1.2.1.2 **RNA viruses**

1.1.2.1.2.1 Orthomyxoviridae – Influenza

Orthomyxoviridae have three genera: A, B and C in which Flu A viruses are further subtyped based on the 2 major antigens: hemagglutinin (HA; H1-H18) and neuraminidase (NA; N1-N11).

Flu A viruses have aquatic birds as their natural reservoir and can cause infections in a number of land and water mammals (e.g. humans, pigs, horses, seals) while Flu B viruses are mainly human pathogens, with rare reports of Flu B virus infection in dogs, cats, swine and seals. Influenza C (Flu C) causes (exclusively) human infections, and appears to be quite rare.

Flu A and B viruses with mutations in the regions of the HA and NA genes can exhibit 'antigenic drift', in which the surface antigens of the virus gradually change, progressively and directionally, to escape immunologic pressure from the host species against parent strains. By this mechanism, Flu A and B viruses can cause yearly epidemics of influenza. Such "antigenic drift' has posed a great challenge for the development of influenza vaccines. Influenza virus strains for vaccines have to be updated yearly to match antigenically with the viruses producing the subsequent epidemic. Currently, seasonal influenza vaccine is a trivalent vaccine or quadrivalent vaccine which contains antigens of the following influenza virus strains: one influenza type A subtype H1N1pdm09 virus strain, one influenza type A subtype H3N2 virus strain, and either one or two influenza type B virus strains. Potential

vaccine viruses for a coming season are derived from the WHO's ongoing worldwide surveillance for influenza viruses [45].

Influenza viruses can also change through "antigenic shift". Antigenic shift only happens in Flu A viruses and is caused by the fact that the influenza virus genome consists of 8 segments, and when 2 different viruses infect the same host, reshuffling (reassortment) of these segments may occur producing new progeny viruses. Antigenic shift may produce new influenza subtypes with HA and NA combinations that have emerged from aquatic birds or pigs. When these "shifts" occur, most people have little or no protection against the new virus. The 2009 pandemic H1N1 virus was the result of several such reassortments [46]. Due to the absence of immunity in the human population, new lineages of Flu A virus emerge every few decades leading to global pandemics [47]. Flu B viruses often co-circulate with Flu A viruses and also cause annual epidemics, but there has not been a reported pandemic related to Flu B viruses.

Severe human infections with animal viruses, especially avian viruses, are known to occur sporadically. H7N3 viruses from seals and H7N7 or H9N2 viruses from birds have caused conjunctivitis and – mostly - mild influenza-like illness in patients in close contact with these infected animals [48]. In contrast, H5N1 avian influenza viruses have caused severe human respiratory illness since 1997 in around 650 people, in Asia and North Africa, with a mortality of over 50%, most of whom reported having close contact with wild birds or domestic poultry. Likewise, the novel avian influenza A H7N9 infections were found in China in 2013 with 137 infected and 45 fatal cases reported to WHO as of 25th October 2013 [49]. The main clinical features among most patients with H5N1 and H7N9 infections are respiratory diseases

resulting in severe pneumonia, respiratory failure and acute respiratory distress syndrome (ARDS).

Detection rates of Flu A and B in children with ARI has been reported at between 1% and 11% [16, 19, 21, 31, 32, 34, 42].

1.1.2.1.2.2 Picornaviridae

1.1.2.1.2.2.1 Rhinoviruses (hRV)

hRV have 99 serotypes and are the most common cause of the common cold, an important illness all over the world with repect to morbidity and economic impact.

Besides causing the common cold, there is evidence showing that hRV is a common cause for hospitalization. hRV can cause bronchiolitis, pneumonia, exacerbations of asthma and other chronic lung diseases, and plays an essential role in driving the infant immune system towards the asthmatic phenotype [50]. Many studies showed that hRV accounted for 4 to 28% of respiratory pathogens detected in ARI in children [16, 32, 36, 42, 51, 52]. Detection rates are usually higher in outpatient than in inpatient studies. High detection rates of this virus were found in studies in Madagascar (20.5%) [36], in the Netherlands (24.1%) [52], and in Khanh Hoa, Vietnam (28%) [51] where hRV was the most [36, 51] or the second most common pathogen in children with ARI [52].

1.1.2.1.2.2.2 Other Picornaviridae

Enteroviruses (EV), which are transmitted by the oral-faecal and respiratory route, are not only an important cause of aseptic meningitis in humans, but are also found in association with the common cold, herpangina in children and acute hemorrhagic conjunctivitis. In recent years, EV was the predominant pathogen detected in large

outbreaks of hand, foot and mouth disease in Asian countries like China and Vietnam. Frequent detection of diverse EV in the respiratory tracts of healthy children suggests that many species may be non-pathogenic 'commensal' agents.

Parechoviruses (PeV), like EV, are another common cause of aseptic meningitis, and have also been frequently detected in children with ARI.

1.1.2.1.2.3 Paramyxoviridae

1.1.2.1.2.3.1 Respiratory syncytial virus (RSV)

RSV is the major cause of bronchiolitis and the most common respiratory pathogen in hospitalised children under 2 years of age. Recently, it has become clear that RSV causes significant morbidity in infants as well as in the elderly [53]. The detection rates of RSV in children with ARI in several previous studies were between 8% and 43% [16, 19, 21, 31, 32, 34, 42].

Almost all children suffer at least one RSV infection by 3 years of age, with half of them infected during the first year of life. RSV is also the predominant pathogen in hospitalised children with respiratory infections in Vietnam [16]. Because of the fact that RSV infections do not give rise to persistent neutralizing antibodies, immunity following primary infection does not prevent secondary or subsequent infections.

There are two subtypes (A and B) of RSV which may co-circulate, with one usually predominating in any given year. No obvious differences in disease severity or pathogenesis have been documented between these two subtypes [54, 55].

1.1.2.1.2.3.2 Human metapneumovirus (MPV)

Retrospective serology has shown that although MPV was first discovered in 2001 [40], it is not a new human pathogen, but has been existent for a long time [56]. This

virus is similar to RSV in terms of the disease it causes, its distribution as well as its seasonality. MPV is currently classified into two subtypes. It causes infections in children under 2 years old and the elderly, and studies suggest that prevalence in children with ARI typically varies from 1% to 10% [16, 19, 21, 31, 32, 34, 42].

1.1.2.1.2.3.3 Parainfluenza viruses (PIV)

PIV have four species: PIV1-4, among which PIV-1, PIV-2, and PIV-3 are important pathogens causing LRI in infants, young children, the elderly, the chronically ill, and the immune-compromised. PIV-3 causes bronchiolitis and pneumonia, mostly in the first two years of life. PIV-1 and 2 typically cause croup in children 2-4 years of age [57]. Documented detection rates of PIV in children with ARI have ranged from 0% to 16% according to many studies [16, 19, 21, 31, 32, 34, 42]

Other Paramyxoviridae such as rubeola *virus* (measles) or the zoonotic nipah virus may also be associated with respiratory disease as part of disseminated infection. Measles has not been recognised as a major cause of LRI, despite the fact that in developing countries, measles accounts for 6–21% of ARI morbidity and 8–50% of ARI mortality [58].

1.1.2.1.2.4 Coronaviridae

1.1.2.1.2.4.1 Coronaviruses (CoV)

CoV are ubiquitous and frequently found in respiratory specimens from children hospitalised with URI, asthma exacerbation, acute bronchiolitis, pneumonia, febrile seizures and croup. Four species of CoV (229E, OC43, NL63 and HKU1) have been detected as the second main cause of the common cold. Reinfection of CoV is common due to rapidly decreasing antibody levels. Previous studies showed that CoV

accounted for about 1% to 10% of respiratory pathogens in children presenting to hospital with ARI [16, 21, 31, 32, 42].

1.1.2.1.2.4.2 Severe Acute Respiratory Syndrome-coronavirus (SARS-CoV)

SARS-CoV was identified in 2002 as the cause of an epidemic of Severe Acute Respiratory Syndrome (SARS) with more than 8,000 cases and 744 deaths all over the world. SARS often started with symptoms of viral infections such as fever and myalgia, but then rapidly progressed to a respiratory syndrome of cough, dyspnoea followed by acute respiratory distress syndrome. SARS was mainly transmitted by the respiratory route, but oral-faecal transmission was also reported.

The precursor virus of SARS is found in wild *Rhinolophus* bats which are considered as the natural reservoir [59]. Civet cats, sold as delicacies in Asian markets, were found to harbour these viruses and provided the opportunity for viral adaptation to humans, which gave rise to intense human-to-human transmission before it was contained by an unprecedented world-wide effort.

1.1.2.1.2.4.3 Middle East Respiratory Syndrome-coronavirus (MERS-CoV)

MERS-CoV infections were first reported in humans in September 2012. As per December 2013, there were 166 cases reported to WHO with a mortality of 43% (71) [60]. MERS-CoV also has its reservoir in bats, and dromedary camels have recently been indentified as an intermediate host [61-63] and there is evidence for camel-to-human transmission of MERS-CoV [64, 65]. Most patients present with respiratory symptoms such as fever, cough, and shortness of breath [66].

1.1.2.2 Bacterial agents

Compared to viruses, bacteria account for a relatively small percentage of cases of ARI. According to Turner et al, bacterial diagnoses were established in about 19% of outpatients with pneumonia [67]. SP is the most frequently isolated pathogen in ARI followed by Hin and *Staphylococcus aureus* [37, 68].

SP, a member of the *Streptococcus mitis* group is the most commonly isolated respiratory pathogen in community acquired pneumonia. SP accounted for almost 42,000 invasive infections in the United States in 2007, leading to approximately 4,500 deaths. SP is a major cause of meningitis, and otitis media, and is also found as a colonizing bacterial species in the oropharyngeal cavities of many asymptomatic carriers [69]. Carriage rates among young children are 30% to 70%, depending on the sampling method, while detection rates in healthy adults are often reported to be below 5% [70-72].

Hin is also found as part of the commensal bacterial flora in the upper respiratory tracts of many healthy individuals. Capsular type b strains of Hin may cause meningitis, epiglottitis, orbital cellulitis, and bacteraemia, particularly in developing countries, where people have not been vaccinated with Hin type b conjugate antigen vaccine. In developed countries, most Hin infections are now caused by non-encapsulated Hin strains including acute conjunctivitis, acute otitis media, acute maxillary sinusitis, acute bacterial exacerbation of chronic bronchitis, and pneumonia. Carriage of encapsulated strains of Hin type b is rare with levels of 2 to 5% of healthy children in the prevaccine era but significantly lower (0.06%) after the paediatric Hin type b conjugate antigen vaccine was introduced in the early 1980s [73, 74].

Bordetella parapertussis (BPp) and *Bordetella pertussis* (Bpt) belong to the genus *Bordetella* and the family *Alcaligenaceae*. Bpt is transmitted by droplets, and with susceptible contacts the transmission rate may reach to 90%. Bpt and BPp cause pertussis, or whooping cough. The typical clinical symptoms of whooping cough found in primary infections of non-vaccinated children include coughing spasms, whooping, and vomiting (paroxysmal phase). In comparison to Bpt infections, BPp diseases are often clinically milder, less vomiting and shorter in terms of duration of coughing. In countries with high coverage of pertussis vaccination, the incidence of pertussis is very low, and the majority of whooping cough cases are now identified in neonates, unvaccinated young infants, older schoolchildren, adolescents, and adults because protection wanes after several years [75]. In Vietnam, the Expanded Programme on Immunization (EPI), which included pertussis vaccination, has been implemented since the early 1980s. In 2012, the coverage rate of pertussis vaccination in Vietnam was almost 96% and the incidence of pertussis was about 0.1 per 100,000 persons [76].

Atypical bacteria include *Mycoplasma pneumoniae* (MP), *Chlamydophila pneumonia* (CPn) and *Chlamydophila psittaci* (CPs), *Coxiella burnettii* and *Legionella* spp. These agents cause syndromes that overlap partially with viral respiratory infection but do not usually cause the typical clinical picture of lobar pneumonia caused by SP and other bacteria and do not respond to beta-lactam agents, hence the name 'atypical'.

Among atypical bacteria, MP has been identified most frequently in children with ARI, at about 2% to 16.2% [21, 42, 77]. MP belongs to the family *Mycoplasmataceae* in the class Mollicutes. In the general population, this bacterium is

the causative pathogen in approximately 20% of all community-acquired pneumonias. MP causes upper as well as lower respiratory tract infections in children over 5 years old and young adults. However, in recent years, MP was also found as a causative pathogen of ARI in children under 5 years of age as well as in older people. The most typical clinical manifestation is tracheobronchitis, often accompanied by URI. Approximately one-third of persons infected with MP develop pneumonia [78, 79].

CPn and CPs are intracellular bacteria and members of the *Chlamydiaceae*. CPn is a common human respiratory pathogen and causes similar clinical manifestations as MP. Clinical manifestations of CPn infections are nonspecific and often not different from those caused by MP and respiratory viruses. CPn was found as the causative pathogen in 10 to 15% of cases of community-acquired pneumonia in adults as well as in children [79].

CPs causes psittacosis, a zoonotic infection related to exposure to birds, presenting with fever, headache and myalgia, hepato- and/or splenomegaly, and may also present with pneumonia [80]. Cardiac complications including endocarditis and myocarditis may occur. Psittacine birds and a wide range of other avian species are the natural reservoir of CPs, which can be transmitted to humans either by direct contact with infected birds or the inhalation of aerosols from nasal discharges and from infectious faeces or feather dust. There is no evidence for human-to-human transmission [81]. In Vietnam, the first five cases with CPs infections were reported in a one-year clinical trial conducted in the National Hospital for Tropical diseases (NHTD), Hanoi from February 2011 to March 2012 [82].

Coxiella burnetii, another intracellular bacterium, is the causative agent of Q-fever, which is an acute febrile illness that is either self-limiting or develops into pneumonia

or endocarditis or a systemic chronic syndrome. Cattle, sheep and goats are the main reservoir and humans can be infected by aerosol inhalation. There is, however, no human-to-human transmission [83].

Legionella pneumophila (LP) belongs to a group of aquatic Gram negative bacteria, which grow densely in warm water and live and multiply within free living amoebae. LP is widespread in plumbing systems, water heaters, warm water spas, and cooling towers. LP causes more than 90% of cases of Legionnaires' disease, a form of bacterial pneumonia which ranges from mild to fatal in severity. The average fatality rate in patients with Legionnaires' disease was recorded at about 12% [84].

1.1.2.3 Co-infections

Current advanced molecular diagnostic techniques make it now possible to rapidly and simultaneously detect multiple pathogens within biological samples.

1.1.2.3.1 **Viral co-infections**

Over the past five years, several studies using multiplex RT-PCR to investigate respiratory pathogens have shown detection of two or more viral pathogens in up to 44% of upper respiratory samples from young children with ARI [16, 19, 21, 31, 32, 34]. High co-detection rates have been reported in pre-school children from low-income families living in Brazil, Vietnam and Jordan [16, 21, 32]. Some authors suggest that co-infection is a risk factor for severe disease. However, there is no real consensus supporting this premise.

1.1.2.3.2 **Bacterial co-infections**

The significance of simultaneous bacterial and viral co-infection in acute respiratory infection is difficult to assess, particularly when analysis is limited to upper airway

secretions. It can be inferred that viral pathogens detected in the upper airways are also in the lower airways of children with chest symptoms, but the same cannot be said for bacteria, where upper airway detection is as likely to be due to asymptomatic nasopharyngeal carriage.

A study from the Gambia of 74 children with community-acquired pneumonia indicated that a third of the 45 children with confirmed pneumococcal pneumonia also had RSV infection [85]. In contrast, in children from Malawi with a high prevalence of HIV, only 9% of those with pneumococcal infection had a viral co-infection (the commonest being with AdV) [86].

1.2 Antibiotic use in acute respiratory infection in children

1.2.1 Antibiotic development and consumption

1.2.1.1 Antibiotic development

The first antimicrobial agent in the world, discovered by Paul Ehrlich and Sahachiro Hata in 1910, was the highly toxic arsenic compound arsphenamine (later called salvarsan), a "magic bullet" for the treatment of syphilis.

Twenty-two years later, in 1932, Gerhard Domagk, a German bacteriologist, discovered another group of antimicrobials: the sulphonamides.

In 1928, Alexander Fleming discovered penicillin from the fungus *Penicillium notatum*. After becoming available for clinical use in the 1940s, penicillin became an outstanding drug in terms of safety and efficacy and helped to save thousands of lives during World War II. The next two decades thereafter were considered the golden age of antimicrobial chemotherapy, when many new classes of antibiotics were developed.

In 1944, Selman Waksman discovered streptomycin, an aminoglycoside active against tuberculosis and many other infections. Thereafter, in the 1950s, chloramphenicol, tetracycline, macrolide and glycopeptides were discovered from soil bacteria.

In 1962, nalidixic acid, a quinolone antibiotic, was introduced for the first time. Cephalosporins were first developed in the 1960s. The first-generation cephalosporins including cephalothin, cephalexin, and cefazolin, which are effective only for Gram-positive bacteria, were introduced in 1962, 1967 and 1970, respectively. Cefaclor, one of the most popular second- generation cephalosporins, was produced in 1977 and became available in the United States 2 years later (in 1979). The third-generation cephalosporins (ceftazidime, cefotaxime, ceftriaxone) which are active against Gram-negative organisms, were developed in 1980s. The fourth- generation cephalosporins including cefpirome, cefepime, and cefclidine have activity against *Enterobacter* spp., *Citrobacter freundii* and *Serratia marcescens* because they are not hydrolysed by their chromosomal beta-lactamase ampC. These were introduced in 1983, 1987 and 1989, respectively. Meanwhile, in the late 1980s, carbapenems and new quinolone antibiotics were developed [87, 88].

The emergence of bacterial resistance against antibiotics was one of the main drivers in the development of new types and classes of penicillins and related antibiotics. Penicillins, cephalosporins (including cephamycins), monobactams and carbapenems together make up the family of beta-lactam antibiotics, based on their structural similarity. One of the major mechanisms of resistance to these beta-lactams is the hydrolysis of the beta-lactam ring by enzymes called beta-lactamases produced by bacteria. Penicillinases are a specific group of the beta-lactamase family. The first

penicillinase was identified in 1940 in *Escherichia coli* strains. In order to combat the rapid spread of beta-lactamase resistance, many new classes of antibiotics have been developed. At the end of 1950s, penicillinase-resistant penicillins such as oxacillin or methicillin were developed. Since 1970, inhibitors of beta-lactamase were obtained with the introduction of clavulanic acid and followed by sulbactam and tazobactam.

1.2.1.2 Antibiotic consumption worldwide

The rapid development of the antibiotic industry has gone hand in hand with the increasing global sales of these drugs. In just two years from 1994 to 1996, the world market for antibiotic increased 28%. In 1995, global spending on antibiotics was more than $1.8 billion and increased to $3 billion by 1998. The total world market for new and currently available antibiotics reached $22.3 billion in 2001. This market climbed to nearly $36 billion in 2008 and further increased to $37 billion in 2009. It is projected to reach $50 billion in 2014 [89].

About 11,900 potential antibiotics have been discovered through 1994. Beta-lactam antibiotics, including the penicillins and cephalosporins are the most commonly used since they are the most effective, have a broad spectrum and are generally safe. The global sales of these drugs showed an average annual growth of 11% from 1986 to 1995, accounting for 60 - 80% of the world antibiotics market [14].

Surveillance of outpatient antibiotic consumption in European countries showed that the volume of outpatient antibiotic use in DDD (Defined Daily Dosage) per 1000 inhabitants per day (DID) increased in most European countries between 1997 and 2003 [90] and this trend continued between 2004 and 2009 [91]. The median use was 19.0 DID and interquartile range 15.1–23.1 DID. The highest consumption was found in Greece (38.6 DID) and the lowest was in Romania (10.2 DID) [90].

Penicillins were the most frequently prescribed antibiotics in all countries, ranging from 29% (Germany) to 66% (Slovenia) of total outpatient antibiotic use [91].

In the cephalosporin group, second-generation cephalosporins are most commonly used, followed by first and third-generation cephalosporins (Figure 1-1) [92].

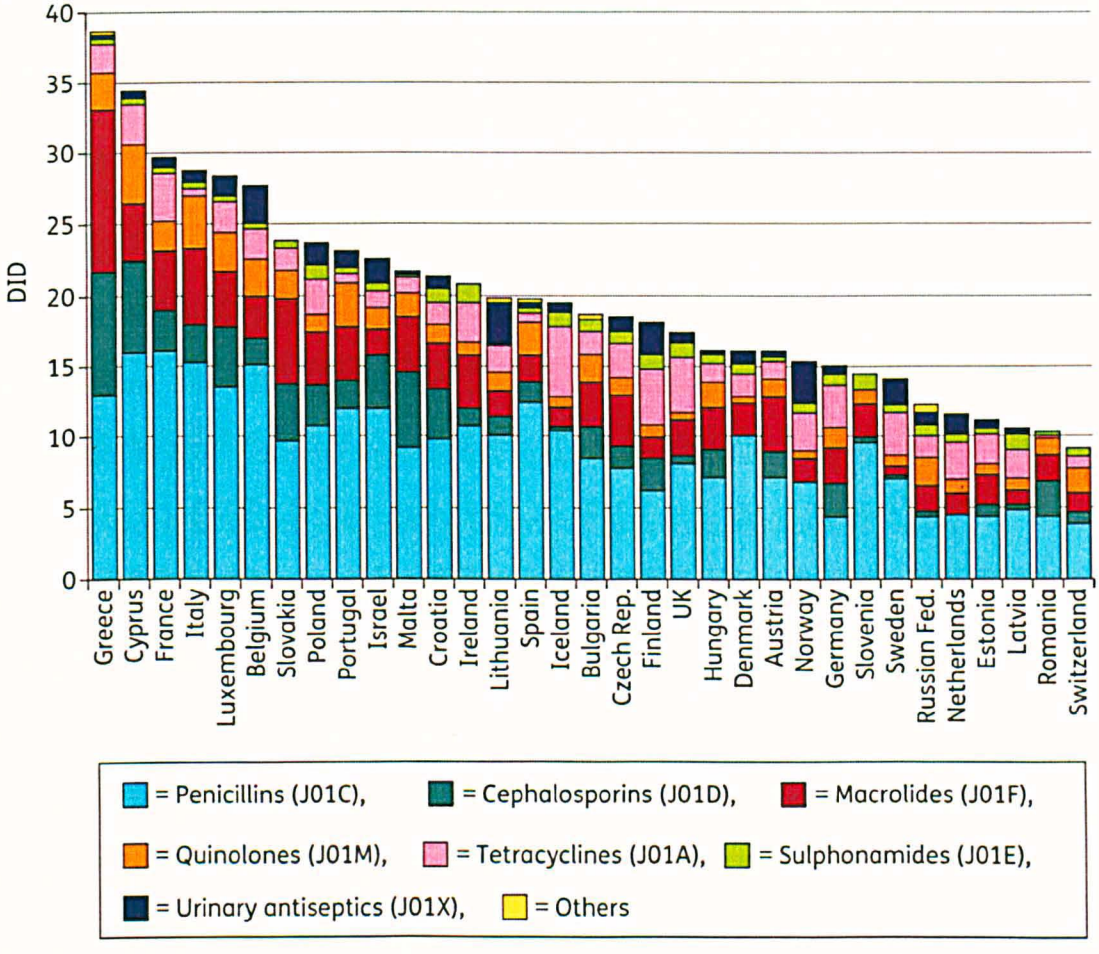

Figure 1-1 Total outpatient antibiotic use in 33 European countries in 2009 in DID (2004 data for Switzerland) [92]

Surveillance of antibiotic use in developing Asian countries is difficult because few datasets are available and antibiotics can be purchased over-the-counter without prescription. A one–year survey in Delhi, India from December, 2008 to November,

2009 showed that total antibiotic use in DID was about 118, 343, and 223 in public facilities, private retail pharmacies, and private clinics, respectively. The rate of using at least one antibiotic was 39.3% (3615/9205 patients) in public facilities, 39.5% (7101/17995) in private retail pharmacies, and 43.4% (2571/5922) in private clinics. In both private pharmacies and private clinics, fluoroquinolones were the most commonly prescribed group of antibiotics, followed by the cephalosporins and the penicillins. In the public sector, the highest consumption was of penicillins and fluoroquinolones, followed by the macrolides, tetracyclines, and cephalosporins [93].

1.2.1.3 Antibiotic Use in Vietnam

In 2009, the Vietnamese Ministry of Health conducted a survey in 100 hospitals (including central general hospitals as well as specialist and provincial hospitals) which showed that antibiotics accounted for approximately 36% of total hospital drug costs. The lowest antibiotic expenditure, 3%, was found in a psychiatric hospital while a paediatric hospital in Ho Chi Minh City had spent the highest budget for antibiotics, at 89%. According to IMS, a commercial organization which tracked the sales of 439 retail drug stores and 62 hospital pharmacies in the 5 largest cities in Vietnam (Ho Chi Minh City, Ha Noi, Da Nang, Hai Phong and Can Tho), oral cephalosporins were the most frequently sold antibiotics at both retail drug stores and hospital pharmacies while the second, third and fourth most commonly sold antibiotics were oral broad spectrum penicillins, macrolides and fluoroquinolones, respectively (IMS data in 2008-2009).

In Vietnam, people have free access to most kinds of (oral) antibiotics, although legislation banning antibiotic purchase without prescriptions has been in place since 2005. Over-The-Counter (OTC) sale of antibiotics is extremely common in Vietnam.

Throughout the country, there were about 39,000 pharmacies (data from 2009) where customers can buy several different kinds of medications, including antibiotics, that normally would require prescriptions by law [94]. People usually prefer to go to pharmacies, rather than clinics or medical settings, to purchase medications in order to save money and time [95, 96]. In most pharmacies, the permanent drug sellers behind the counter are often not the pharmacists, but sales clerks. Pharmacists often rent out their license and work elsewhere. At the pharmacies, customers describe their signs and symptoms and the sales clerks, with little or no medical training, give patients separate pills/medications without name, package or package inserts. Therefore, customers often do not know the names or the kinds of medications they are using.

In order to ensure proper, safe, and costly uses of medication, the Ministry of Health has implemented many measures since the 1990s. In 1996, the National Drug Policy was issued and highlighted the importance of appropriate use of antibiotics, limiting its use to prescription only. In 1997, all government hospitals were required to establish a Drug and Therapeutic Committee (DTC) with responsibilities related to medication and antibiotic use: building standard treatment guidelines, evaluating rational use of medications including antibiotics, monitoring antibiotic resistances in the hospitals, and providing medical staff with drug information. In 2005, the Drug Law was promulgated and clearly stated that antibiotics were to be given only with prescriptions [97]. In outpatient settings, the 2007 Regulation No 04/2008/QD-BYT stipulated that only physicians at legal health care centres could prescribe antibiotics. This regulation also stated that physicians should not prescribe antibiotics to meet patients' (inappropriate) requests [98]. Despite these measures, in reality, customers

can still purchase antibiotics very easily without prescriptions in almost all pharmacies in Vietnam.

1.2.1.4 Antibiotic use in Children's Hospital 1

Children's Hospital 1 (CH1) is the largest referral hospital for children in south and central Vietnam. The number of outpatients attending the hospital is approximately 1,600,000 yearly (from statistical data of CH1, 2011). The total cost for medications in the outpatient clinic was 60 billion VND (3 million USD) in 2011, of which antibiotics accounted for 43% (26 billion VND – 1.3 million USD). Beta-lactams constituted the greatest proportion of total antibiotic expenditure: second-generation cephalosporins accounted for 36.3% of total antibiotic costs, followed by amoxicillin–clavulanic acid (32%), third-generation cephalosporins (23.7%), and first-generation cephalosporins (2.3%). The least commonly used antibiotic class in outpatient clinic in CH1 were fluoroquinolones (0.56%). Macrolides made up about 5%.

Table 1-3 Antibiotic expenditure in outpatient department in CH1 in 2011

Antibiotics	Expenditure (USD)	Percentage (%)
Amoxicillin	4,110	0.3
Amoxicillin – clavulanic acid	419,588	31.9
First generation cephalosporins	29,523	2.25
Second generation cephalosporins	476,520	36.3
Third generation cephalosporins	311,368	23.7
Fluoroquinolones	7,352	0.56
Macrolides	65,390	4.98
Total	1,313,852	100

(Source: statistical data of CH1, 2011)

1.2.2 **Management of acute respiratory infection in children**

Children with very severe disease or severe pneumonia must be admitted to a hospital to receive intensive treatment and care, while most patients with moderate to mild pneumonia or non-pneumonia are given home care. There are many standard treatment guidelines or textbooks for ARI management in children such as WHO's integrated management of childhood illness (IMCI) [12], Nelson's Paediatric Textbook [99], WHO's ARI programme [8], or evidence-based reviews from the Cochrane library [100-102]. According to these, antibiotics are indicated only in individuals with pneumonia, severe pneumonia or otherwise very severe disease, and ear infections. For children with only a cough or a common cold (no pneumonia), no antibiotics are recommended but rather symptom relief measures are recommended such as antipyretics to reduce high fever or cough syrup to soothe the throat and alleviate the cough.

1.2.2.1 Antibiotic treatments in acute respiratory infection in children

Although many previous studies have shown that viruses are the dominant aetiologic agents in children with ARI [1-4, 7, 16, 36, 52], the rates of antibiotic use directed at bacterial pathogens are very high, particularly in developing countries. For instance, 87.8% of outpatient children with ARI were prescribed antibiotics in a cross-sectional study in Iran in 2006 [103]. High rates of antibiotic prescription were also found in developed nations like the United States of America, where 75 – 80% children with respiratory infections were given antibiotics, according to a study by Steinman et al in the period from 1994 to 1998 [104]. In Australia, antibiotic prescription rate was 57% and 73% for urban and rural patients with URI, respectively, from 1990 to 1995 [105].

In medical settings, which have very stringent antibiotic use policies and must strictly follow treatment guidelines for ARI, significantly lower rates of antibiotic prescription were found. In Iran, the antibiotic prescription rate among 0-15 year-olds with ARI was rather low (33%) in the outpatient clinic of a children's hospital in Tehran after implementing and monitoring a new ARI treatment algorithm in 2007 [106]. In the Netherlands, the rate of antibiotic use among 0–15 year olds with respiratory infections attending general practitioners' clinics in 2000 was approximately 25% [107]. At the Children's Hospital, Islamabad, Pakistan, antibiotic use declined noticeably from 54.6% of ARI outpatients in 1989 to 30.2% in 1992 ($P < 0.0001$) as a result of strictly following the WHO standard treatment for ARI in which children with viral ARIs were not given antibiotics [108].

1.2.2.2 Antibiotic use in acute respiratory infections in children in Vietnam

With support from WHO and UNICEF, the Ministry of Health in Vietnam has issued national guidelines for childhood infection management including acute respiratory infections. For infections in the community, the 2006 guideline recommended penicillin or amoxicillin for treating pneumonia. As an alternative, amoxicillin with clavulanic acid or a second or third generation oral cephalosporin was advised. A macrolide should be added to the regimen if atypical bacteria were suspected. At the hospital level, the guideline advised use of amoxicillin (with or without clavulanic acid) or a second or third generation cephalosporin or a macrolide for community – acquired pneumonia.

From 2006 onwards, most health stations and centres in Vietnam, that see patients under 15 years old, have implemented the WHO's IMCI programme. This programme guides healthcare workers and mothers on how to assess and manage childhood

diseases including respiratory infections. Co-trimoxazole, amoxicillin or erythromycin are recommended only for pneumonia, while there is no indication for antibiotics if patients have only cough or common cold without signs of pneumonia (fast breathing or chest indrawing). However, over-prescription of antibiotics can be seen clearly in Vietnamese children with ARI, most of which are caused by viruses, and varied from 80% to 91%, according to several previous studies in Ba Vi [95, 96]. In a study by Larsson et al, conducted in Ba Vi in 2007, despite the existence of guidelines, 91% of children with cough or common cold were treated with antibiotics, of whom only 20% consulted physicians, 11% bought antibiotics by themselves without any consultation, and 67% consulted pharmacy staff, of whom only 27% had proper knowledge about antibiotic use. As a result, 74% of children were found to carry resistant pathogens (SP, Hin and *Moraxella catarrhalis*) in respiratory samples [95]. The most frequently dispensed antibiotics were ampicillin or amoxicillin (49%), oral first-generation cephalosporins (27%), co-trimoxazole (11%), and macrolides (3%).

1.2.2.3 Antibiotic prescriptions for acute respiratory infections in Children's Hospital 1

In CH1, ARI was the most common disease in the outpatient department. Data from 2011 showed that ARI accounted for almost 30% of total outpatient visits. There is a "drug and therapeutic committee" in CH1, which has implemented a number of measures to control inappropriate drug usage, particularly for antibiotic use in ARI. CH1 has promulgated its own ARI therapeutic guidelines (Appendix G) based on the National Therapeutic Guideline of the Ministry of Health (Appendix H) and the WHO's IMCI guidelines (Appendix F). Training about the hospital treatment

guidelines has been provided annually for all physicians in the hospital. However, the rate of doctor adherence to ARI therapeutic guidelines is low; in current practice, almost 90% of children with ARI presenting to the outpatient department in CH1 are prescribed antibiotics (unpublished data).

1.2.3 Antibacterial agents in paediatrics and mechanisms of resistance

1.2.3.1 Definition and mechanisms of antibiotic resistance

Resistance to antibiotics is a naturally occurring biological phenomenon. It refers to the ability of the microorganism to multiply in the presence of antibiotic concentrations that are higher than in humans receiving therapeutic doses [109]. According to WHO, "antimicrobial resistance (AMR) is resistance of a microorganism to an antimicrobial medicine to which it was previously sensitive" [110].

The launch of any new antimicrobial agent into clinical practice has (often within a few years) been followed by the detection of resistant strains of microorganisms. The key driver of resistance emergence is the selective pressure associated with exposure of bacteria to antibiotics. Any antibiotic use, regardless of the level of use, increases the likelihood of resistance [109].

There are several mechanisms that lead to antimicrobial resistance: (a) permeability changes in the bacterial cell membrane, which prevent antimicrobial access to target sites; (b) active efflux of the antimicrobial from the cell through pump molecules; (c) mutation in the target site; (d) enzymatic modification or degradation of the antimicrobial; and (e) acquisition of alternative metabolic pathways to those inhibited by the drug. Resistance may be caused by spontaneously occurring mutations but

numerous bacterial antimicrobial resistance phenotypes also result from acquisition of external genes that may provide resistance to an entire class of antimicrobials. These genes are frequently associated with large transferable extra-chromosomal DNA elements called plasmids, that may carry additional mobile DNA elements such as transposons and integrons [111].

1.2.3.2 Beta-lactam antibiotics

Beta-lactam antibiotics that contain a Beta-lactam ring - a lactam with a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom - are considered well tolerated, safe, and efficacious drugs against most bacterial agents in childhood infections. Beta-lactam antibiotics include penicillins, cephalosporins, carbapenems, and monobactams. These drugs are bactericidal agents against both Gram positive and Gram negative bacteria. These agents kill bacteria by inhibiting the synthesis of the peptidoglycan layer of the bacterial cell wall. The synthesis of peptidoglycan chain is catalysed by a transpeptidase enzyme called penicillin-binding protein (PBP). Beta-lactam antibiotics have a similar structure to the normal building blocks of the bacterial cell wall and by irreversibly binding to these transpeptidases, beta-lactam antibiotics inhibit the synthesis of the cell wall, resulting in cell lysis.

Reported mechanisms of resistance to beta-lactams include the hydrolysis of beta-lactam ring by beta-lactamases from bacteria, and structural alteration of transpeptidase by mutations in PBPs (penicillin-binding proteins). The production of beta-lactamases in bacteria is encoded by genes that are either chromosomal or plasmid-borne, by which means they can be transferred to other bacteria (mostly by conjugation) [112]. Very soon after the resistance to first line antibiotics was observed, many new beta-lactam antibiotics with broader spectrum were developed to

withstand the hydrolytic action of these beta-lactamases. Beta-lactamase inhibitors (which often also have a beta-lactam structure and some antibiotic activity), have evolved in nature and are now also used as additive drugs in dual preparations to act as competitive inhibitors of beta-lactamase activity and to restore the antimicrobial activity of beta-lactam antibiotics against resistant bacteria. The combination of beta-lactamase inhibitors with several penicillins has improved the availability of antibiotic agents available for paediatric patients. Amoxicillin-clavulanic acid, the most widely used combination in paediatrics, is efficacious against methicillin-susceptible *S. aureus*, beta-lactamase–positive Hin and many anaerobes [111, 113].

The other mode of resistance is alteration in cell-wall structural enzymes – PBPs that are present in almost all bacteria and act in the final stages of cell wall synthesis. Beta-lactam antibiotics, as a result, cannot bind effectively to the altered enzyme's active site, and hence, cannot interfere with the bacterial wall synthesis. Typical bacteria that can acquire this mode of resistance include methicillin-resistant *S. aureus* (MRSA) and penicillin-resistant SP [111].

1.2.3.3 Extended Spectrum Beta Lactamases (ESBLs)

Extended-spectrum beta-lactamases (ESBLs) are able to hydrolyze a wider spectrum of beta-lactam antibiotics than the simple parent beta-lactamases from which they are evolved. ESBLs have inhibiting activity against beta-lactam antibiotics containing an oxyimino-group such as oxyimino-cephalosporins (ceftazidime, ceftriaxone, cefotaxime) as well as oxyimino-monobactam (aztreonam). However, ESBLs are not active against cephamycins (cefoxitin, cefotetan, cefmetazole and flomoxef) and carbapenems [114].

ESBLs are produced by a vast number of Gram-negative rods, of which the majority belong to the *Enterobacteriaceae* family, but they have also been found in non – *Enterobacteriaceae*, among which *Pseudomonas aeruginosa* is the most important [115].

To date, more than 200 ESBLs have been characterised. The most prevalent ESBL types have evolved from the narrow spectrum TEM and SHV beta-lactamases. TEM-1 was discovered in 1965 from an *E.coli* isolate in a patient named Temoniera in Athens, Greece (hence named TEM). SHV (sulfhydryl variable) is a plasmid-mediated beta-lactamase commonly found in *Klebsiella* spp. and *E.coli* [114]. CTX-M is the second largest group of ESBLs. The name CTX reflects its activity against cefotaxime. These enzymes inactivate cephalothin better than benzylpenicillin and preferentially hydrolyze cefotaxime over ceftazidime [114]. The CTX-M enzymes, originally described in South America, Asia, and Eastern Europe, have spread worldwide [116].

OXA-type lactamases are so named because of their oxacillin-hydrolyzing abilities. Only some OXA types have ESBL activity, with resistance against cefotaxime and sometimes ceftazidime and aztreonam.

There are a variety of other beta-lactamases that are plasmid-mediated or integron-associated enzymes. VEB-1 was first discovered in a single isolate of *E. coli* from a 4-month-old orphan child who was previously admitted to an intensive care unit in Vietnam [117]. VEB-1 provides resistance against ceftazidime, cefotaxime, and aztreonam. The gene mediating VEB-1 was found to be plasmid encoded and these plasmids also confer resistance to non-beta-lactam antibiotics. The PER-type-ESBLs share approximately 25 to 27% homology with the known TEM- and SHV type

ESBLs. PER-1 beta-lactamase was first detected in *P. aeruginosa* strains isolated from Turkey, and inactivates penicillins and cephalosporins.

ESBLs are commonly plasmid-mediated beta-lactamases. Plasmids responsible for producing ESBLs often co-carry a number of other genes that encode resistance to other antibiotic classes such as aminoglycosides [118-120] or fluoroquinolones [120-127].

Recently, transferable resistance against the last effective class of beta-lactam antibiotics was detected. The New Delhi Metallo-beta-lactamase-1 (NDM-1) is encoded by the plasmid borne bla_{NDM-1} gene, and has been found in Gram negative bacteria such as *Enterobacteriaceae* and renders them resistant to a broad range of beta-lactam antibiotics including carbapenems. Since first detected in a Swedish patient of Indian origin in 2008, these enzymes have been detected worldwide including in Europe, America, Africa, Asia and Australia. Loss of the carbapenems as a therapeutic option may cause bacteria to become untreatable, and herald a return to the pre-antibiotic era [128-132].

1.2.3.4 Fluoroquinolones

The fluoroquinolones are broad-spectrum bactericidal agents that act as inhibitors of DNA supercoiling – a specific bacterial/prokaryotic way of storing DNA - by binding to topoisomerases or gyrases, bacterial enzymes which are present in most bacteria, but not in humans. They have bactericidal activity against gram-positive organisms (including some penicillin non-susceptible pneumococci and MRSAs); against gram-negative bacteria (including the *Enterobacteriaceae, M. catarrhalis, beta-lactamase–producing* Hin, *Neisseria* spp. and *P. aeruginosa*), atypical or intracellular organisms

(including *Mycoplasma* spp. and *Chlamydophila* spp., LP, *Ureaplasma urealyticum*) and *Mycobacterium* spp.

Resistance to fluoroquinolones is regulated by three mechanisms: (1) target mutations in bacterial genes encoding for gyrase and topoisomerase; (2) reduced permeability of the bacterial cell wall; and (3) energy-dependent efflux pumps [111]. An important fourth mode of resistance is plasmid-mediated and was first unveiled in 1998 in *Klebsiella pneumoniae* isolates [127]. The plasmid-encoded protein accounting for resistance, named qnr, binds to and protects both DNA gyrase and topoisomerase IV from inhibition by fluoroquinolones [133]. Currently three families of qnr are recognised: qnrA, qnrB and qnrS [134], among which qnrA is most common [135].

Another mode of plasmid transferable quinolone resistance was recently described: the enzyme aminoglycoside acetyltransferase that inhibits activity of ciprofloxacin by N-acetylation of its piperazinyl amine. This enzyme is encoded by a variant of the aac(6')-Ib acetyltransferases (see below) [136].

Plasmid mediated quinolone resistance (PMQR) genes may be co-carried on plasmids along with ESBLs and other resistance genes producing multi-drug resistant strains.

1.2.3.5 Aminoglycosides

Aminoglycosides are bactericidal inhibitors of bacterial protein synthesis that act by binding to 16S rRNA and inhibiting initiation of translation and translational fidelity. They have broad spectrum activity against gram-positive and gram-negative bacteria, but in clinical practice are mostly used intravenously against gram-negative infections or topically as broad spectrum agents for eye infections. Gentamicin, tobramycin, and amikacin are among the three most commonly prescribed aminoglycosides in the paediatric population [113]. Aminoglycosides resistance is primarily the result of

spontaneously evolving structural point mutations in the target genes, and the production of modifying enzymes that can phosphorylate, adenylate or acetylate these agents [111]. These aminoglycoside resistance genes may co-localise on the same plasmids as ESBLs and PMQR genes. [118, 137-140].

1.2.3.6 Macrolides

The macrolides act against bacterial protein synthesis by attaching to the tRNA binding site on the 50S subunit and causing the tRNA molecules to dissociate from the ribosomes [111]. They have activity against SP, *S. aureus*, *M. catarrhalis*, and *Streptococcus pyogenes* and the newer macrolides (azithromycin and clarithromycin) against Hin. Azithromycin has the broadest activity, including against *Shigella* and *Salmonella*. Other organisms that could be inhibited by the macrolides include atypical and intracellular pathogens, such as MP, *U. urealyticum*, *Legionella* spp., *Chlamydia* and *Chlamydophila* spp. and *Mycobacterium* spp.. In paediatrics, with the exception of group A beta-haemolytic *Streptococcus* pharyngitis, the macrolides are not widely used [113]. There are numerous resistance mechanisms against macrolides, a detailed description of which is beyond the scope of this thesis.

1.2.4 Consequences of antimicrobial resistance

Antimicrobial resistance results in prolonged infection duration with greater risks of death as a consequence of unresponsiveness to the medications. Additionally, prolongation of the duration of infections caused by resistant bacteria may lead to pathogen transmission to other patients and/or transmission of resistance factors to other bacteria. Furthermore, longer duration of treatment and the requirement for

more expensive antimicrobials leads to major rises in health-care costs and creates financial burdens for patients, hospitals and societies [110].

A study in a Chicago teaching hospital in the US found that the extra medical costs for a patient who acquired antibiotic-resistant infections ranged from almost $19,000 to around $29,000 while the duration of hospital stay for patients with antibiotic-resistant infections was prolonged by 6.4 to 12.7 days. The death rate among patients with antibiotic-resistant infections was 2-fold higher than that in patients with infections that were antibiotic susceptible. The societal costs for 188 patients with antibiotic-resistant infections in this hospital in 2008 were $10.7-15.0 million. [141]. In a nested, matched cohort study of patients admitted to Beth Israel Deaconess Medical Centre between 1994 and 1997, the mean attributable hospital charge due to infections of antimicrobial resistant *Enterobacter* was $29,379 and the median attributable duration of hospital stay was 9 days. In comparison to people who became infected with antibiotic-susceptible organisms, patients who acquired antibiotic-resistant *Enterobacter* had much higher costs (around 1.5-fold increase), higher length of hospital stay (more or less 1.5-fold increase) and higher mortality [142, 143]. Likewise, Schwaber et al compared the outcomes of patients with bacteraemia caused by ESBL-producing bacteria and those with bacteraemia due to non ESBL producers and found an increase in hospital stay (1.57-fold increase), total costs (1.57-fold increase) as well as significantly higher mortality among patients infected with ESBL producers [144].

1.2.5 **Interventions to contain antimicrobial resistance**

WHO has provided a list of interventions to slow the emergence and decrease the spread of antimicrobial-resistant microorganisms: (1) lessening the disease burden

and the spread of infection; (2) augmenting access to appropriate antimicrobials; (3) improving use of antimicrobials; (4) empowering health systems and their surveillance capabilities; (5) accomplishing regulations and legislation; (6) encouraging the development of appropriate new drugs and vaccines. Among these interventions, improving antimicrobial use must be a key component in efforts to contain resistance.[109].

In many European countries with well regulated health systems, restricting antibiotic use and improving prescriptions to contain antimicrobial resistance have been performed by a multipronged approach involving several simultaneous measures: education about antibiotic use for patients and treating doctors; monitoring of antibiotic consumption and resistance; development of standard treatment guidelines; promulgations of regulations and rules for appropriate antibiotic usage [145].

In developing countries, where most healthcare settings have a poor infrastructure, limited regulations and treatment guidelines, and inadequate health education, approaches to decreasing antibiotic resistance present a great challenge. Lack of knowledge, and absence of standard guidelines and regulations for antibiotic use are big problems in the developing world. There is a misconception that all infections and ailments can be cured by antibiotics. Parents believe antibiotics are "wonder bullets" and expect to get them when ill in almost all infections. Doctors often feel safer prescribing children with antibiotics instead of symptom-relief treatments for ARI, most of which are caused by viruses, to prevent possible secondary bacterial infections despite evidence showing no value of such prophylaxis. Pharmacists easily dispense antibiotics without prescription for financial reasons. Therefore, training on the rational use of antibiotics not only for medical staff but also for non-professionals

is a key measure [145]. Many previous studies have shown the effectiveness of patient education in improving appropriate antibiotic use in children [146-152]. Education programmes or campaigns that use a multifaceted approach and target both patients and physicians have provided solid improvements in the appropriate use of antibiotics [148]. Training on the use of C-reactive protein (CRP) testing and enhanced physician-patient communication for clinicians have also demonstrated positive effects on rational use of antibiotics in patients with ARI [153, 154]. WHO have recommended the simultaneous implementation of multiple measures including both education and diagnostic interventions. Rapid bedside diagnostic tests, or white blood cell count and CRP could be applied in order to assist the treating physicians to make better clinical decisions. Regulations and rules should be developed to assure the appropriateness of antibiotic use [145].

1.3 Antibiotic use and selection of resistant gut bacteria

1.3.1 Human gut flora: composition and functions

Humans are naturally colonised with numerous microorganisms that reside on the skin, mucosa and gastrointestinal tract; these are referred to as the human microflora or microbiome. The human digestive system is the natural habitat for the largest bacterial community. In a normal individual, the gastrointestinal tract contains approximately 100 trillion bacteria with 300-500 different species, 80% of which have not yet been well characterised [155]. The human gut contains more bacterial cells than the total number of cells in the entire human body (estimated at about 37.2 trillion) [156, 157]. The anaerobic bacteria are predominant in human gut flora and outnumber the aerobic bacteria such as *Enterobacteriaceae* and *Enterococcus* spp.

[158]. *Enterobacteriaceae* are an important family of Gram negative bacilli in the human intestine and consist of many genera such as *Escherichia, Shigella, Salmonella, Citrobacter, Klebsiella, Enterobacter, Serratia, Proteus, Yersinia* and others.

The composition of the microorganisms varies greatly from person to person and is influenced by dietary habits, diarrhoeal illness, and antibiotic treatment [159]. A large number of studies have revealed the relation between microflora and host health. Bacteria in the normal intestinal flora are able to break up certain food components, produce vitamins K and group B [160, 161], play a role in absorption of calcium, magnesium, iron [162], stimulate the production of natural antibodies and produce digestive enzymes to ferment non-digestible dietary residues and salvage the energy [162]. In addition, gut flora can enhance intestinal epithelial cell growth and differentiation [163, 164]. Most importantly, normal microbes provide a barrier effect and prevent colonization of exogenous microorganisms as well as potential pathogens within the gut [165]. It is suggested that there is a complex interdependence between the normal gastrointestinal bacteria and the host immune system [166, 167]. Disorders of the microflora can lead to changes in the immune system that may play an essential part in the genesis of certain pathogenic conditions [168]. A study by Moore et al showed that a high risk of colon tumour was linked with the presence of about fifteen bacterial taxa from the human gut flora, whereas low risk was significantly associated with the presence of five other genera [159].

Resident bacteria of the gut flora also have a key role in the pathophysiology of inflammatory bowel diseases [169] [23] [170, 171]. An altered intestinal flora may contribute to intestinal diseases such as infectious diarrhoea in children and irritable

bowel syndrome [172-174] and a number of extra-intestinal diseases [175] [176]. Diarrhoea and dysentery are the most common intestinal infections that may be caused by a few members of *Enterobacteriaceae* including *E.coli, Shigella species, Salmonella* species, and *Yersinia enterocolitica.* The majority of extra-intestinal infections involve a small number of species: *E.coli, K. pneumoniae, Klebsiella oxytoca, Proteus mirabilis, Enterobacter aerogenes, Enterobacter cloacae,* and *S. marcescens,* which may cause nosocomial infections such as urinary tract infections, respiratory infections, septicaemia, or wound infections.

It is now widely accepted that our intestinal flora also act as a reservoir of antibiotic resistance genes. A study in 162 adult individuals (85 Danish, 39 Spanish and 38 Chinese individuals,) in 2013 revealed that they carried a total of 1,093 antibiotic resistance genes among 4.1 million gut genes. In comparison to other natural environments such as soil, oceans and lakes, antibiotic resistance genes are much more abundant in human intestinal flora [177].

The human gut flora play an essential role in trafficking antibiotic resistance genes. There is not only the exchange of resistance genes among intestinal bacteria, but also an interaction between resistant gut bacteria and bacteria passing through the alimentary tract, causing these bacteria to acquire and transmit antibiotic resistance genes, especially under selective pressure, e.g. in hospital settings with high use of third generation cephalosporins.

This resistance gene transfer has also been demonstrated in several experiments in laboratory animals [178-181].

1.3.2 Biological cost of antibiotic resistance and constraints to resistance development

Bacterial fitness is defined as the ability to survive and reproduce in a given ecosystem [182]. Antibiotics act upon many important biological functions of bacteria such as cell wall synthesis, regulation of chromosome super coiling, RNA transcription and protein synthesis. Bacteria may develop resistance by point mutations in the target genes, altering their function. In the process of developing resistance, bacteria usually suffer a fitness cost. Plasmid carried resistance genes will be easily transferred under antibiotic pressure, but bacteria may get rid of them when that pressure is gone. The frequency and rates of acquisition and dissemination of antibiotic resistance are inversely related to the biological cost and directly associated with the volume of antibiotic use. In other words, a reduction of antibiotic use should in theory result in a decrease in the frequency of resistant bacteria [182] [183].

The biological cost of antibiotics resistance makes it possible for susceptible bacteria to outcompete resistant bacteria if the selective pressure of antibiotics is lifted. However, there have been several findings suggesting that resistant bacteria can reduce the fitness cost, and therefore maintain resistance by mechanisms such as compensatory mutations [184-194], cost-free resistance mutations [195, 196] and co-selection phenomena of resistance [197]. Additional fitness-compensatory mutations have helped reduce the biological costs for resistant bacteria allowing them to compete successfully with sensitive strains in an antibiotic-free environment leading to the persistence of several resistant genes for years [182, 184, 185, 189, 198, 199]. Similar phenomena have been observed for plasmid encoded antibiotic resistance. Although plasmids carrying resistance genes come with a biological cost to their

bacterial hosts, plasmids have evolved mechanisms to ensure their successful 'transmission' to daughter cells. Compensatory mutations of the bacterial host genome reducing the fitness costs of plasmid replication have also been reported [200-202].

In a stable microbial community such as the human gut flora, wild type bacteria are dominant as their physiology has adapted to that system for millions of years. Under the increasing pressure of antibiotics, susceptible bacteria are inhibited and resistant cells may dominate the population. However, once the antibiotic pressure is removed, resistant organisms may disappear due to their attenuated fitness associated with the resistant phenotype. As a result, the microflora will be restored to its original state. On the other hand, if the fitness cost is affordable or negligible, although a large number of resistant bacteria are replaced by wild type strains, they may be maintained in the population (partial restoration). This process will repeat itself if the use of antibiotics is resumed.

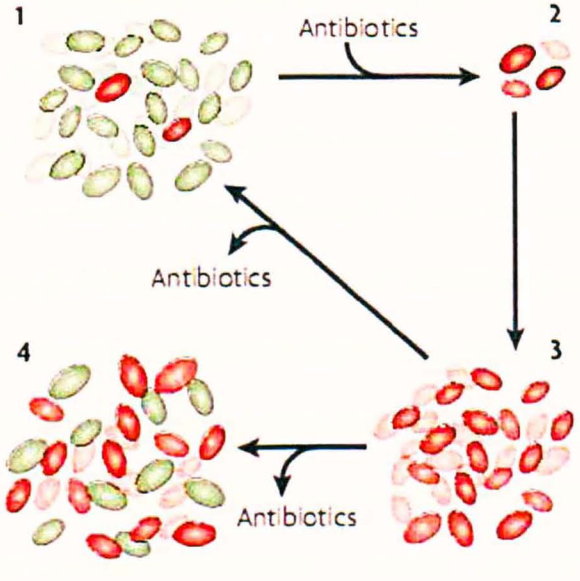

Figure 1-2 Understanding antibiotics resistance as a colonization factor [203]

Red: antibiotic resistance phenotype

Green: wild type

[1]. When a bacterium acquires an antibiotic resistance phenotype, it must compete with wild-type bacteria. In general, it is not able to survive unless it has higher biological fitness.

[2]. Under selective pressure of antibiotics, resistant bacteria will be dominant.

[3]. When selective pressure is lifted, if the fitness cost of antibiotic resistance phenotype is not affordable, resistant bacteria will be replaced by wild-type bacteria and the microflora is restored to its baseline state.

[4]. In contrast, if the fitness cost of antibiotic resistance phenotype is affordable, resistant bacteria may be maintained in the ecosystem. If antibiotics are reused, the process can repeat itself.

1.3.3 **Selection of resistant intestinal bacteria following antibiotic use**

Antibiotics, when reaching the human colon, can suppress the normal susceptible microorganisms leading to the overgrowth of pre-existing, natural resistant microorganisms like yeast and *Clostridium difficile* or new colonization with resistant bacteria. There have been several studies that found an increase in the number of colonizing and/or resistant bacteria in the human gut flora after oral intake of antibiotics belonging to the penicillin class such as ampicillin [204] or amoxicillin with/without clavulanic acid [204-208], An increased number of resistant intestinal *Enterobacteriaceae* and *Enterococcus* spp. have also been demonstrated after adult patients used oral antibiotics belonging to the second or third- generation cephalosporin class [205, 206, 209]. Similar overgrowth of resistant colonizing bacteria in gut flora was also found after intake of other antibiotics such as

doxycycline or co-trimoxazole in a community-based study in southern Germany. This study also showed that two weeks after stopping antibiotic therapy the prevalence of resistant bacteria returned to the original (pre-treatment) levels. This demonstrated the short -term impact of antibiotics on the number of colonizing and resistant bacteria in human intestinal microflora [210].

Most previous studies performed in adult healthy volunteers with small sample sizes have focused on the changes in the number of colonizing bacteria and the resistance of bacteria to only one antibiotic they studied, but did not assess changes in the fraction of resistant bacteria in gut flora to other commonly used antibiotics. There have not been any studies exploring the effect of antibiotics prescribed in children with mild ARI not requiring hospital admission on the resistance of human gut flora.

1.4 Summary

From all the medical literature reviews mentioned above about acute respiratory infections, I have gleaned the following summary and questions:

1. ARIs are among the most common diseases in children with higher morbidity and mortality in developing countries. The epidemiology, aetiology, and clinical features of ARI have been well described in textbooks and many previous studies around the world. The presentation and treatment characteristics of ARI have not been defined in Vietnamese children, particularly in children with mild ARI presenting to our outpatients clinics which make up the most part of ARI cases.
2. As regards aetiology, which are the viral and bacterial pathogens responsible for mild ARI in children in Vietnam?
3. Antibiotics are easily purchased and used without prescriptions in Vietnam. Antibiotic use by self-treatment or visiting pharmacies rather than by consulting

doctors is very common in the community. The true rate of antibiotic use in patients before coming to health care centres remains unknown as people often are not told or do not remember which medications they used. What can be a reliable method to identify the use of antibiotics without prescription?

4. In health care settings in Vietnam, the magnitude and spectrum of antibiotics prescribed by paediatricians for outpatients with mild ARI have not been well studied. What proportion of children presenting at an outpatient clinic in Vietnam with non-severe ARI will have antibiotics prescribed and what proportion of these can be classified as appropriate or inappropriate?
5. Overuse of antibiotics will inevitably have consequences for the resistance rates among human pathogens, but what is the effect of antibiotic misuse on the antibiotic resistance of the patients' normal gut flora?

1.5 Objectives, outline and structure of the thesis

To address the above questions, I conducted two prospective descriptive studies: one in ARI patients and the other in healthy children. In the study of ARI patients, I enrolled children with mild ARI presenting to the outpatient clinic of CH1 to describe the epidemiology, presentation, prescribed treatment and clinical characteristics of non-severe ARI in Vietnamese children. Rectal swabs were taken before and after using antibiotics to assess changes in bacterial resistance in the gut flora. A high performance liquid chromatography (HPLC) technique was developed to determine the presence of antibiotics in patients' urine samples before the time point of enrolment. For investigating ARI aetiology, respiratory specimens of both ARI patients and healthy children were obtained and tested by multiplex real time PCR.

The following were the specific study objectives of the thesis:

1. To assess the epidemiology, clinical features, treatment characteristics and outcomes of acute respiratory infections in outpatients (chapter 3).
2. To identify the viral and bacterial respiratory aetiologic agents in children with mild ARI in comparison to a control group of healthy children in Ho Chi Minh City, Vietnam (chapter 4).
3. To assess the short-term effect of antibiotic use on the selection of resistant bacteria in rectal swabs (chapter 5).
4. To assess antibiotic use at enrolment by parent interviews and high performance liquid chromatography of urine samples from children with ARI (chapter 6).
5. To quantify inappropriate antibiotic use in outpatient acute respiratory infections in CH1, Vietnam (chapter 7).

Chapter 2

MATERIALS AND METHODS

This chapter describes the research setting, study design, and the participants in the two studies. Data and sample collection, laboratory experiments and data analysis are also depicted here.

2.1 Research settings

Patient enrolment and sample collection were carried out at the Outpatient Department of CH1, Ho Chi Minh City. Laboratory analysis was conducted at Oxford University Clinical Research Unit, Viet Nam.

2.1.1 Children's Hospital 1 (CH1)

Located in Ho Chi Minh City, CH1 is the biggest referral hospital for children in southern and central Vietnam with 1400 beds and 22 clinical wards. CH1 receives patients aged between 0 and 15 years old and provides almost all paediatric internal specialities such as pulmonology, cardiology, neurology, urology, nephrology, emergency, intensive care, endocrinology, infectious diseases, haematology, neonatology as well as paediatric surgical specialities. The number of in-patients admitted to CH1 is approximately 95,000 per year, 32% of which are for respiratory infections.

In the outpatient clinic, there are 54 examination rooms, of which 30 are used for general and internal diseases and the rest of the 24 rooms are for specialities such as pulmonology, cardiology, neurology, nephrology, endocrinology, infectious disease, haematology, and neonatal-perinatal diseases.

There are more than 1,600,000 outpatient visits yearly, nearly 50% of which are for acute respiratory infections. Approximately 60% of patients at CH1 come from southern and central provinces of Vietnam (from yearly statistical data of CH1, 2011)

We conducted two studies in the outpatient department. The first study in children with acute respiratory infections was implemented in two examination rooms for respiratory diseases which receive approximately 45,000 patients annually. The other study in healthy children was implemented in the vaccination room where 20,000 children are seen annually for consultations and vaccinations.

2.1.2 **Oxford University Clinical Research Unit (OUCRU)**

For almost two decades, the Oxford University Clinical Research Unit, as part of the Oxford Centre for Tropical Medicine of the Oxford Centre for Tropical Medicine, has developed the infrastructure and capacity to perform clinical trials and basic scientific research in Vietnam, as well as other parts of South and South East Asia (www.oucru.org). Established in 1991 with support from the Hospital for Tropical Diseases (HTD, a tertiary referral hospital in Ho Chi Minh City), the Health Services of Ho Chi Minh City and the Wellcome Trust, OUCRU has developed strong links not only with HTD, but also other hospitals in Ho Chi Minh City, Hanoi and other regions of Vietnam. In February 2006, an OUCRU office in Hanoi was opened. Infectious disease topics include malaria, typhoid and enteric infections, dengue, tuberculosis, HIV and opportunistic infections, infections of the central nervous system, zoonoses, influenza and emerging viral infections.

In this study, all laboratory work was performed at the Microbiology and Molecular Diagnostic Laboratories at OUCRU-VN.

2.2 STUDIES

2.2.1 Study on antibiotic use in acute respiratory infections among outpatients (03AV)

2.2.1.1 Study design and setting

This study was a prospective descriptive study of the clinical characteristics, aetiology, antibiotic use and selection of resistant gut bacteria in children presenting to the outpatient department of CH1 with acute respiratory infections. We chose the outpatient department at CH1 for this study because CH1 is a tertiary referral hospital for children in the southern and central provinces of Vietnam and its number of roughly 800,000 outpatients with respiratory infections annually is most probably representative for the population of outpatient children with ARIs in Ho Chi Minh City as well as the southern Vietnam. In the outpatient department, the two respiratory examination rooms were chosen to enrol patients for this study.

2.2.1.2 Objectives of this study

(1) To describe the epidemiology, clinical features, treatment characteristics and outcomes of ARI in outpatients.

(2) To identify viral and bacterial respiratory pathogens in children with mild ARI

(3) To assess the short-term effect of antibiotic use on the selection of resistant bacteria in the gut microflora.

(4) To determine antibiotic use prior to enrolment by means of parent interviews and high performance liquid chromatography of urine samples from children with ARI

(5) To quantify the inappropriate antibiotic use in outpatient ARI.

2.2.1.3 Sample size calculation

For this observational study, although no formal sample size calculation was undertaken, the following factors were taken into account: collection of samples was spread over a period of one year to correct for seasonal variations in respiratory infections; therefore, at a rate of 10 patients per week, 500 patients were enrolled for the year. Five hundred patients with an antibiotic prescription rate of 85% (results from a cross-sectional study in CH1 in 2000- unpublished data) and a determined viral aetiology in 60% (4), will enable assessment of antibiotic overuse (0.85 x 0.60 = 0.51) with an acceptable precision of 0.04 (0.47-0.55).

2.2.1.4 Inclusion and exclusion criteria

Patients attending the respiratory examination room at the outpatient department in CH1 were eligible for enrolment into the study if they were under 16 years of age, had a diagnosis of ARI (see below), were not admitted to the hospital, their parents or legal guardians gave informed consent and if they lived in Ho Chi Minh City and agreed to return for follow up visit after 1 week and 1 month (if requested).

ARI was defined as having at least one of the following symptoms as the chief complaint lasting shorter than 5 days: cough, sore throat, runny nose or nasal congestion.

Patients were not eligible for enrolment if they had any underlying illness (except asthma), or previous admission to the hospital within the past 3 months (in any hospital or health centre).

2.2.1.5 Enrolments:

Patients were enrolled from between 9th February 2009 and 4th February 2010. To enrol 500 patients in one year, which translates to around 10-12 patients a week, patients were screened every day from Monday to Thursday (Monday 8-9am, Tuesday 9-10am, Wednesday 10-11am, Thursday 1-2pm) until at least two patients (and a maximum of 4 patients) per day met the eligibility criteria and were enrolled to the study.

2.2.1.6 Data collection:

For each eligible participant, a case report form (CRF) (Appendix B) was filled in with patient information including general demographics and past medical history, current clinical signs and symptoms and antibiotic use. All medications which doctors prescribed for patients on presentation were also recorded in the CRFs.

For the first and second follow-up (6-8 days and 28-42 days, respectively, after presentation), data recorded included clinical signs and symptoms on the day of follow-up and antibiotics the patients had used.

The information contained within the CRFs was then entered into a computerised database.

2.2.1.7 Sample collection:

The following specimens were collected from patients:

- On the day of enrolment (day 1): 1 nasopharyngeal aspirate (NPA), 1 nose swab, 1 throat swab, 1 rectal swab, 1 fingerprick blood sample and 1 urine sample.
- On the first follow-up (day 6-8): 1 rectal swab, 1 urine sample.
- On the second follow-up (day 28-42): 1 rectal swab.

NPAs were collected using N-PAK nasopharyngeal aspirate kits (M-Pro, LLC, Annandale, MN, USA). Respiratory swabs were collected in Viral Transport Medium (VTM). Rectal swabs were collected in 1ml of 0.9% saline solution. Urine was collected in a sterile plastic container. Blood was collected with a glass capillary after puncture of the skin using a genie lancet (Becton Dickinson, Franklin Lakes, NJ, USA) and was spotted on 903 protein saver cards (Whatman, Singapore). Cards were allowed to dry on a dry rack for 4 hours, and stored in a ziplock plastic bag.

All specimens were temporarily kept in a refrigerator in CH1 and transferred daily to OUCRU for processing, storage and further laboratory investigations. NPAs and respiratory swabs were stored at -80 °C until use. Rectal swabs were processed immediately for bacterial culture (see below). Urine and blood samples were stored at -80 °C until use.

2.2.1.8 Endpoints

2.2.1.8.1 Primary endpoint was the appropriateness of antibiotic use in the studied population.

2.2.1.8.2 Secondary endpoints were:

- Presentation and treatment characteristics of ARI in outpatient settings.
- Clinical outcome at follow-up.
- Aetiology of ARI.
- Fractions of resistant bacteria in rectal swabs at presentation and at follow-up.
- Diversity of resistance associated genes in bacteria from rectal swabs.
- Presence of antibiotics in urine on admission.

2.2.2 Study on the presence of respiratory bacteria and viruses in healthy children (01RS)

2.2.2.1 Study design and setting

This study was designed as a prospective descriptive study to identify the carrier rate for respiratory 'commensal' viruses and bacteria among healthy children in Ho Chi Minh City, to serve as a control group for multiple studies on respiratory infections in both out- and inpatient children in CH1. This control population will enable comparisons of incidence rates and copy numbers of respiratory viruses and bacteria in children with and without disease. Healthy children were approached at the vaccination outpatient department at CH1.

2.2.2.2 Objective of study

To assess the presence of respiratory pathogens in healthy children

2.2.2.3 Duration of study

The patient enrolment phase in this study was carried out over a full year from the 6th December 2010 to 1st December 2011.

2.2.2.4 Inclusion and exclusion criteria

According to an enrolment schedule, each month we enrolled 5 children under 1 year of age, 10 children between 1 and less than 5 years of age, and 5 children 5 years of age or older.

All children under 16 years of age presenting to the vaccination outpatient department in CH1 were eligible for enrolment if they were healthy enough to be eligible for vaccination on the day of enrolment and if their parents or legal guardians gave informed consent. Children were excluded if they had fever, underlying illness

(except asthma) or had been admitted to hospital within the previous 3 months (in any hospital or health centre).

Mild respiratory symptoms for which no medical advice was sought were not considered an exclusion criterion and were recorded in the CRF.

2.2.2.5 Enrolment

With each child enrolled into the study, a CRF was used to collect general information such as sex, date of birth and history of antibiotic use. Swabs from the nasal cavity and the throat were collected in VTM or RNAlater. All specimens were temporarily stored in a refrigerator at CH1 before daily transport to OUCRU for processing, storage at -80 °C and further analyses.

2.3 Ethics

All study protocols were reviewed and approved by the Scientific and Ethics Committee of Children's Hospital 1, the Health Service of Ho Chi Minh City, and the University of Oxford Tropical Research Ethics Committee (OxTREC) (Appendix E).

2.4 Laboratory experiments

2.4.1 Molecular diagnosis of respiratory pathogens

2.4.2 Multiplex real-time (RT) PCR for respiratory viruses

A published multiplex real-time PCR was implemented and validated at OUCRU to detect the presence of 14 respiratory viruses in respiratory specimens [211].

2.4.2.1.1 Clinical samples

Respiratory specimens from two studies were used. These were nasal swabs and throat swabs (03AV and 01RS), and NPAs (03AV only). For each patient, nasal

swabs and throat swabs were combined 1:1 before further processing, as this was shown to be beneficial for diagnostic yields in earlier studies [212].

2.4.2.1.2 **Control strains**

2.4.2.1.2.1 RNA internal control

2.4.2.1.2.2 Equine Arteritis virus (EAV)

EAV is an RNA virus classified under the *Arteriviridae* family, and is a causative agent of viral arteritis in horses. EAV was used as a non-competitive RNA-virus internal control. It was included in the PCR assay prior to extraction at a standard concentration yielding crossing point (Cp) values of 30-35 to monitor the process of extraction, reverse transcription, amplification, and detection, as previously described [213].

2.4.2.1.2.3 Positive controls

External positive controls for the Multiplex Real Time PCR consisted of 14 plasmids (pCR II TOPO TA, Invitrogen, Carlsbad, CA, USA) into which the specific target fragment of each virus was cloned. In each of the 4 PCR reactions of the multiplex PCR (see below) 4 corresponding plasmids at 100 copies per reaction were used.
The negative control was water.

2.4.2.1.3 **Nucleic acid extraction**

In the 03AV study, nucleic acids were extracted from 100µl of swabs in VTM or NPAs and eluted in 60µl of elution buffer provided in the Easy MAG kit, by means of an automated commercial GuSCN-based method, according the instructions of the manufacturer (Easy MAG 2.0, bioMérieux, Marcy l'Étoile, France). For the 01RS study extraction was done on the MagNA Pure 96 system, another commercial

GuSCN based extraction platform, using the total nucleic acid kit (Roche, Mannheim, Germany). On this platform, nucleic acids are extracted from 100 µl and eluted in 100 µl of molecular grade water. Different platforms were used because the analysis of samples from these two studies was performed at different times and the molecular diagnostic facility had changed the extraction platform. The biochemistry used by both platforms, their extraction efficiency and subsequent qualitative and quantitative results of this and other PCRs were very similar (this was evaluated for many different assays before implementation of the novel platform).

2.4.2.1.4 **Reverse Transcription (RT)**

In this study, the real time PCRs were performed in two steps. In the first step reverse transcription of RNA into cDNA was done separately from the other reaction steps and processed outside the PCR machine. Viral RNA was reversely transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, California, USA) and random hexamers (Roche, Mannheim, Germany). Each 20µl reaction mixture contained 5µl extracted RNA, 4µl of 5X RT-buffer, 0.5mM of each dNTP (Roche), 2ng random hexamer, 10mM DTT (Invitrogen), 1UI of RNAse inhibitor and 2 UI RT Superscript III. The cDNA synthesis was performed using an Eppendorf Master thermocycler gradient system (Perkin-Elmer Corporation, Foster City, California, USA) under the following conditions: 10 min at 25°C, 60 min at 50°C and 15 min at 75°C.

2.4.2.1.5 **Multiplex real time PCR**

The multiplex PCR was targeted at 14 different respiratory viruses: RSV A/B, FluA, FluB, AdV, EV, MPV, CoV (CoV-229E, OC43, HKU1, SARS CoV & NL63), hRV

A, B and C, PIV-1, 2 3, and 4, PeV and BoV [211, 214]. The PCR was performed in 4 tubes on a Roche LC480 II Thermocycler (Roche Diagnostics, Penzberg, Germany). A total volume of 20µl contained 5µl of a cDNA, 10µl of 2X Probes Master (Roche Diagnostics, Penzberg, Germany), 900nM of primer (each), and 200nM of probe (each). Thermocycling settings were as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles each consisting of 15 s at 95°C and 1 min at 60°C. The multiplex real-time RT-PCR results were semi-quantitatively expressed as Cp-value, as defined specifically by the LightCycler 480 II software.

Primers and probes for the different targets were pipeted in 4 tubes as below:

M1 : FluA – FluB – AdV – EV

M2 : RSV A/B – MPV – hRV – EAV

M3 : PIV 1 – PIV 2 – PIV 3 – PIV 4

M4 : CoV1 – CoV2 – BoV – PeV

Primers were manufactured by Sigma Proligo (Singapore). Probes containing Minor Groove Binders (MGBs) were manufactured by Applied Biosystems Inc (ABI, Foster City, CA, USA), probes containing LCRED 610, 670 or CYAN500 were manufactured by Tib Molbiol (Tib, Berlin, Germany), and probes containing only HEX or FAM by Sigma Proligo.

Table 2-1 Primers and probes for multiplex PCR of 14 respiratory viruses

Primers/probes	Sequences
FluA - F primer	GACAAGACCAATCCTGTCACYTCTG
FluA - R primer	AAGCGTCTACGCTGCAGTCC
FluA - probe	LCRED610-TTCACGCTCACCGTGCCCAGTGAGC-BBQ
FluB - F primer	TCGCTGTTTGGAGACACAAT
FluB - R primer	TTCTTTCCCACCGAACCA
FluB - probe	CYAN500-AGAAGATGGAGAAGGCAAAGCAGAACT-DB
AdV - F primer	CAGGACGCCTCGGRGTAYCTSAG
AdV - R primer	GGAGCCACVGTGGGRTT
AdV - probe	LCRED670-CGGGTCTGGTGCAGTTTGCCCGC-BBQ
EV - F primer	CAGGACGCCTCGGRGTAYCTSAG
EV - R primer	GGGATTGTCACCATAAGCAGCC
EV - probe	6-FAM-GCGGAACCGACTACTTTGGGT-MGBNFQ
RSV - F primer	ATGAACAGTTTAACATTACCAAGT
RSV - R primer	GTTTTGCCATAGCATGACAC
RSVA - probe	LCRED610-TGACTTCAAAAACAGATGTAAGCAGCTCC-BBQ
RSVB - probe	LCRED610-TTATGACATCAAAAACAGACATAAGCAGCTCAG-BBQ
MPV - F primer	AGCTTCAGTCAATTCAACAGAAG
MPV - R primer	CCTGCAGATGTYGGCATGT
MPV - probe	LCRED670-TGTTGTGCGGCAGTTTTCAGACAATGC-BBQ
hRV - F primer	AGSCTGCGTGGCKGCC
hRV - R primer	ACACGGACACCCAAAGTAGT
hRV - probe	CYAN500-TCCTCCGGCCCCTGAATGYGGCTAAYC-DB
PIV1 - F primer	ATCTCATTATTACCYGGACCAAGTCTACT
PIV 1- R primer	CATCCTTGAGTGATTAAGTTTGATGAATA
PIV1 - probe	CYAN500-AGGATGTGTTAGAYTACCTTCATTATCAATTGGTGATG-DB
PIV2 - F primer	CTGCAGCTATGAGTAATC
PIV2 - R primer	TGATCGAGCATCTGGAAT
PIV2 - probe	LCRED610-AGCCATGCATTCACCAGAAGCCAGC-BBQ
PIV3 - F primer	ACTCTATCYACTCTCAGACC
PIV3 - R primer	TGGGATCTCTGAGGATAC
PIV3 - probe	LCRED670-AAGGGACCACGCGCTCCTTTCATC-BBQ
PIV4 - F primer	GATCCACAGCAAAGATTCAC
PIV4 - R primer	GCCTGTAAGGAAAGCAGAGA
PIV4 - probe[=]	HEX-TATCATCATCTGCCAAATCGGCAA-BHQ

Primers/probes	Sequences
CoV1 - F primer	GGTGGYTGGGAYGATATGTTACG
CoV1 - R primer	KRTTTGGCATAGCACGATCACA
CoV1 - probe	6-FAM-ATGTTGACAAYCCTGTWCTTATGGGTTGGG-MGBNFQ
CoV2 - F primer	GCTRAGCATGATTTCTTTACTTGG
CoV2 - R primer	CARTYTTKTTCATCAAAGTTACGCA
CoV2 - probe	6-FAM-CAGARTCATTTATGGTAATGTTAGTAGACA-MGBNFQ
BoV - F primer	CAAATCTCTTCTGGCTACACG
BoV- R primer	CTCTGCGATCTCTATATTGAAGG
BoV- probe	LCRED670-ATGTTGCCGCCAGTAACTCCACC-BBQ
PeV - F primer	CTGGGGCCAAAAGCCA
PeV - R primer	GGTACCTTCTGGGCATCCTTC
PeV - probe[±]	6-FAM-AAACACTAGTTGTAWGGCCC-MGBNFQ
EAV - F primer	CATCTCTTGCTTTGCTCCTTAG
EAV - R primer	AGCCGCACCTTCACATTG
EAV- probe	Fam-CGCTGTCAGAACAACATTATTGCCCAC-BHQ3

2.4.2.2 Real-time PCRs for detection of atypical bacteria

These were used to detect the presence of genetic material from 6 different atypical bacteria associated with respiratory infections: BPp, BPt [215, 216], CPn [217], CPs [218], LP and MP [219]. Protocols were all developed in the Academic Medical Centre, Amsterdam, the Netherlands. All were validated for clinical diagnostic use. Except for the LP PCR, all were published or based on published assays.

2.4.2.2.1 Quality control

Six plasmids (pCR II TOPO TA) into which the specific target sequence for each of the PCR assays was cloned were used as external positive controls. Each PCR reaction used positive control plasmid at 100 copies per reaction.

Water was used as negative control for extraction and amplification.

2.4.2.2.2 Reagents and components of real-time PCR premix

Primers and probes

Table 2-2 Primers and probes for multiplex PCR of respiratory atypical bacteria

Primers/probes	Sequences
BPp F	CACCGCCTACGAGTTGGAGAT
BPp R	CCTCGACAATGCTGGTGTTCA
BPp probe	6-FAM-ACAGCCCACAGGCGGAGAT-MGBNFQ
Bpt F	GATTCAATAGGTTGTATGCATGGTT
Bpt R	TTCAGGCACACAAACTTGATGGGCG
Bpt probe	6-FAM-TTGAGAAACTGGAAATCGC-MGBNFQ
LP F	GACTCTTTACCAAACCTGTGGHC
LP R	CGCGGAAATGTTTCACTTCT
LP probe	6-FAM-TGGCGACTATAGCGATT-MGBNFQ
MP F	CACCCTCGGGGGCAGTCAG
MP R	CGGGATTCCCCGCGGAGG
MP probe	6-FAM-ATTGTCCCTGCTGGTCCATCCC-MGBNFQ
CPn F	TTCGGTTGAGGAAGAGTTTATGCG
CPn R	AATCCGCCTAGACGTCATCG
CPn probe	6-FAM-TCAGCTTGTTGGTGGGGTAAAAGCCC-TAMRA
CPs F	CGCTCTCTCCTTACAAGC`C
CPst R	AGCACCTTCCCACATAGTG
CPs probe	6-FAM-AGGGAACCCAGCTGAACCAAGTTT-TAMRA

2.4.2.2.3 Procedure

To carry out real time PCR, a 20μl reaction mixture containing extracted DNA and PCR mix was pipeted into the wells of a 96-well PCR plate. For all PCRs 2x Probes Master Mix (Roche) was used and primers and probes were added at the following concentrations: for BPp, BPt and MP: primers at 500nM, probes at 250nM; for LP: primers at 900nm, probe at 250nM; for CPs and CPn: primers at 700nm and probe at 300nM. In the assays to detect both *Chlamydophila* species 8μl of extracted DNA was

added; in all the other assays 5µl was added. Molecular grade water was added to reach a volume of 20µl for all assays.

The PCR plate was then placed in the LightCycler instrument to run the PCR programme. Thermocycling settings were as follows: 10 minutes at 95°C, followed by 40 cycles each consisting of 15 seconds at 95°C and 1 minute at 62°C for both *Chlamydophila* assays and 65°C for all other assays. Results were semi-quantitatively expressed as Cp-value, as defined by the LightCycler 480 II software.

2.4.2.3 Real-time PCR for detection of typical bacteria

Two real-time PCRs were used to detect genetic material from typical respiratory bacteria SP (*ply*) and Hin type b (*bexA*) [220]. These assays had been set up and validated at OUCRU for diagnostics of pathogens of bacterial meningitis in earlier studies [221, 222].

2.4.2.3.1 Internal control

Phocid Herpes Virus is a herpes virus of seals and was used as a non-competitive DNA-virus internal control, which is included in the PCR assay prior to extraction at a standard concentration yielding Ct or Cp values of 30-35 to monitor the process of extraction, reverse transcription, amplification, and detection, as previously described [223].

2.4.2.3.2 External positive control

Reference strain ATTC 49619 was used as external positive control for SP PCR, and a clinical isolate from the hospital for tropical diseases, Ho Chi Minh City, for Hin type b PCR. DNA from these isolates was added prior to amplification at a standard

concentration yielding Cp-values of 30-35. Water was used as negative extraction and amplification control.

2.4.2.3.3 **PCR**

Assays were performed on a DNA Engine Peltier Thermocycler and Chromo 4 Real-time PCR system detector (Bio-Rad, Hercules, CA, USA).

PCR reactions with a total volume of 25 µl were prepared in 96 well plates consisting of 5 µl of extracted DNA, 3.5 mM $MgCl_2$ (Qiagen, Hilden, Germany), 1 U Hot Start Taq DNA polymerase and 1x buffer (Qiagen), 200 µM of dNTPs each (Roche Diagnostics, Mannheim, Germany), 400nM of primer and 100nM of probe for target and internal control (Sigma Proligo, Singapore). Molecular grade water was added to reach a 25µl volume.

Table 2-3 Primers and probes for PCR of typical bacteria

Target	Primer sequence
PhHV F	GGGCGAATCACAGATTGAATC
PhHV R	GCGGTTCCAAACGTACCAA
PhHV probe	CY5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ3
SP F	TGCAGAGCGTCCTTTGGTCTAT
SP R	CTCTTACTCGTGGTTTCCAACTTGA
SP probe	FAM-TGGCGCCCATAAGCAACACTCGAA-TAMRA
Hin F	GGCGAAATGGTGCTGGTAA
Hin R	GGCCAAGAGATACTCATAGAACGTT
Hin probe	FAM-CACCACTCATCAAACGAATGAGCGTGG-TAMRA

Thermocycling settings were 15 minutes of heating at 95 °C followed by 40 cycles of 30 seconds of denaturation at 95 °C, 30 seconds of annealing at 60 °C, and 30 seconds of elongation at 72 °C. Fluorescence was measured during the annealing phase.

Samples were considered positive when the fluorescent signal crossed the threshold (Cp-value) before 35 cycles. Samples positive at cycle 35-40 were repeated.

2.4.2.4 Statistical analysis

All variables of interest were analysed and summarised by group (age, diagnosis, single infection or co-infection). For descriptive statistics, prevalence and percentage were used for categorical variables while mean and standard deviation, or median and interquartile range (IQR) were simple measures for normal or non-normal continuous data.

Comparisons of epidemiological and clinical characteristics among age groups or infection groups (no pathogen, one viral infection, one bacterial infection and co-infection) were examined by means of Fisher's Exact test for categorical data and the Kruskal-Wallis test for continuous data. The differences in the aetiology between two groups (ARI patients versus healthy children or nasal pharyngeal aspiration versus nasal – throat swab), were compared using Chi-square test or Fisher's Exact test (when one or more of the expected count is less than 5) for categorical variables and Mann-Whitney U test for continuous variables. In order to assess agreement in aetiology detection between two methods of sampling (NTS versus NPA) or in determination of antibiotic use between parent interviews and urine tests, a Kappa measure of agreement was used.

All statistical tests were performed as two-tailed tests at 5% significance by IBM SPSS Statistics version 20.

2.4.3 Assessing the fraction of antibiotic resistant bacteria in patients' gut flora

2.4.3.1 Materials

2.4.3.1.1 Clinical samples

Rectal swabs were collected from all patients on study day 0 and 7 and for a small number of patients [n=37] on day 28. Samples were taken according to written SOPs and were only acceptable if visible faecal material was attached. Swabs were resuspended directly in a fixed volume (1 ml) of 0.9% NaCl solution and stored in the refrigerator (4 °C) until daily shipment to OUCRU, where samples were processed on the same day (within 24 hours).

2.4.3.1.2 Strains and isolates

The pansensitive *E. coli* J53-AzideR and characterised resistant strains from the Hospital for Tropical Diseases microbiology laboratory (*K. pneumoniae* VS2692, *K. pneumoniae* VS1813, *Pseudomonas* spp. UV1381, and *Salmonella* EG 240) were used for quality control assessments of antibiotic containing agars used (see below). Minimum inhibitory concentration (MIC) values are indicated in Table 2-6.

2.4.3.1.3 Agar plates

MacConkey (MC) agar, a medium for selection and differentiation of Gram-negative bacteria depending on their ability to ferment lactose, was used to culture *Enterobacteriaceae*.

MC agar plates without antibiotics or with added tetracycline, amoxicillin, amoxicillin-clavulanic acid, ceftazidime, ciprofloxacin, trimethoprim/sulfamethoxazole, gentamicin or meropenem were used according to a concentration of half the MIC for resistance (0.5MIC) as indicated in Table 2-5.

These antibiotics were chosen because they were reported belonging to the most commonly prescribed classes of antibiotics for ARI in hospitals and by pharmacies in the region (see Table 1-3 and Appendix A) and/or were of specific interest.

Nutrient agar (NA) was used for culture of quality control strains.

Table 2-4 Typical components of MacConkey Agar (OXOID)

COMPONENTS	CONCENTRATION
Peptone	20g/l
Lactose	10g/l
Bile salt	5g/l
NaCl	5g/l
Neutral red	0.075g/l
Agar	12g/l
pH 7.4±0.2	

Table 2-5 Antibiotic plates containing antibiotics at Laboratory Standards Institute (CLSI) 0.5 MIC for *Enterobacteriaceae* of each antibiotic

Name of drug	Class	Brand	Concentration
Tetracycline	Tetracyclines	Sigma	4µg/ml
Amoxicillin	Extended spectrum Penicillin	Fluka	8µg/ml
Amoxicillin-clavulanic acid	Extended Spectrum Penicillin+βlactamase inhibitor	Fluka + SmithKline Beecham Pharmaceutical	8/4µg/ml
Ceftazidime	3rd generation cephalosporin	Sigma	2µg/ml
Ciprofloxacin	Fluoroquinolone	Fluka	1µg/ml
Trimethoprim + Sulfamethoxazole	Folic acid synthesis inhibitors	Sigma	2/38µg/ml
Gentamicin	Aminoglycoside	Sigma	4µg/ml
Meropenem	Carbapenem	Sigma	4µg/ml

2.4.3.2 Stool processing

Faecal swabs were transported to OUCRU daily and were processed the same day. Samples were vortexed and six tenfold dilutions were made. 50µl of each was plated on MacConkey agar (MC) plates and incubated overnight at 37°C. The next day, lactose fermenting *Enterobacteriaceae* were identified as large circular smooth pink colonies on MC agar and counted in order to obtain the desired dilution range which rendered a colony count in the range of 20 to 200 colonies per plate for further experiments. If the desired range was not reached, further dilutions were made until 20-200 colonies grew per plate.

In these experiments only lactose fermenting *Enterobacteriaceae* that were characterised as round smooth pink colonies on MC agar were taken into account and counted. In the following chapters, when we refer to *Enterobacteriaceae* counts, we mean lactose fermenting *Enterobacteriaceae*.

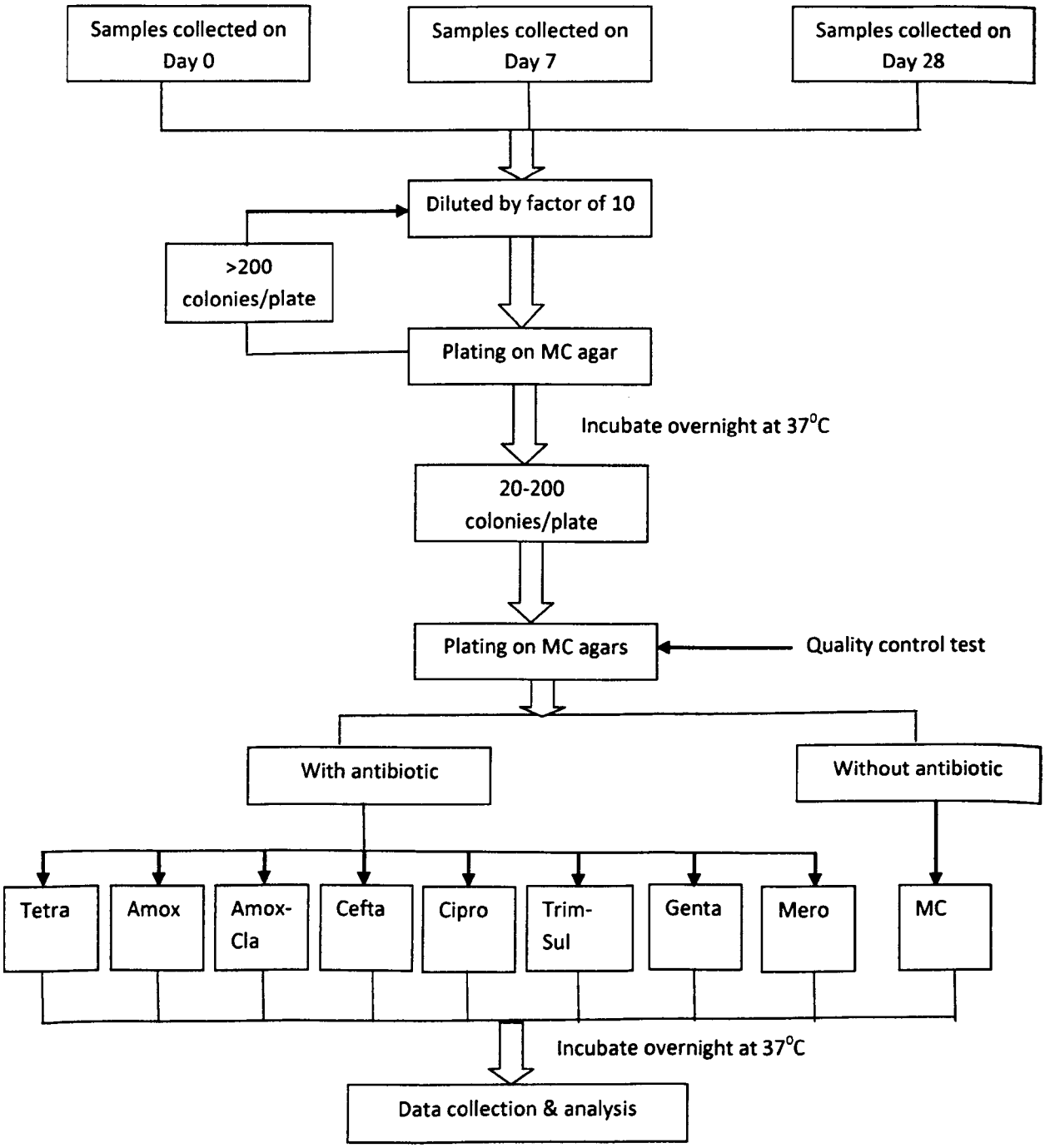

Figure 2-1 Flow chart for stool processing

Tetra: tetracycline; Amox: amoxicillin; Amox-Cla: amoxicillin –clavulanic acid; Cefta: ceftazidime; Cipro: ciprofloxacin; Trim-Sul: trimethoprim/sulfamethoxazole; Genta: gentamicin; Mero: meropenem; MC: MacConkey

2.4.3.3 Quality control

Quality control strains were grown overnight on NA and suspended to an optical density of 0.5 McFarland (assessed using the Vitek Colorimeter, bioMérieux, Marcy l'Étoile, France), diluted 10e5 fold and plated on the different MC agars with and without antibiotics. For every new batch the pan-sensitive and resistant organisms were tested for absence and presence of growth on plates with the corresponding antibiotic, respectively.

Table 2-6 Minimal inhibitory concentration of quality control strains

	E. coli J53Azi	*K. pneumoniae VS 2692*	*K. pneumoniae VS 1813*	*Pseudomonas* spp. *UV 1381*	*Salmonella EG 240*
MC					
Tetracycline	1				>256
Amoxicillin	6				12
Amoxicillin-clavulanic acid	4				12
Ceftazidime	0.38	>256			
Ciprofloxacin	0.008		>32		
Trimethoprim + Sulfamethoxazole	0.064				>32
Gentamicin	0.125				8
Meropenem	0.25			>256	

2.4.3.4 Statistical analysis

Colony counts were log10 (n+1) transformed, and the fraction of resistant *Enterobacteriaceae* for each antibiotic was calculated by the log transformed ratio of numbers of colonies on plates with and without antibiotics. The differences between the fractions at day 0 and 7, and at day 0 and 28 were assessed using the Wilcoxon signed-rank test.

2.4.4 Determination of antibiotics in the urine of children with ARIs by High performance liquid chromatography (HPLC)

In this study, HPLC assays were developed and used for assessment of the presence of six antibiotics in urine using a sample pre-treatment procedure (Solid phase extraction –SPE) as described below and an appropriate running time.

2.4.4.1 Experiments

2.4.4.1.1 Instrumentation

The HPLC system used in this study is the Lachrom Elite – Hitachi (Merck–Hitachi Japan) consisting of an organiser, an auto sampler L-2200, 2 pumps L-2130, a column Oven L-2350 and a Diode Array Detector (DAD) L-2455. The data integration was performed using EZchrom Elite version 3.18 HPLC System Manager Software (Merck–Hitachi Japan).

The chromatographic separation was performed on a 5 µm LichroCart 250x4.6 mm Purosphere RP-18 end-capped, equipped with a 5 µm guard column LichroCart 4x4, RP-18e (Merck, Darmstadt, Germany) for cephadroxil, cefaclor, cephalexin and cefixime; and a 5 µm LichroCart 125x4 mm Purosphere RP-8 end-capped, equipped with a 5 µm guard column LichroCart 4x4, RP-8e (Merck, Darmstadt, Germany) for amoxicillin and cefuroxime.

2.4.4.1.2 Solid phase extraction (SPE) system and other equipment

The SPE process was performed on ISOLUTE SCX 50 mg/1 mL and C8 100mg/mL, cartridges or 96 fixed well plates (Biotage AB, Uppsala, Sweden) (Figure 2-2).

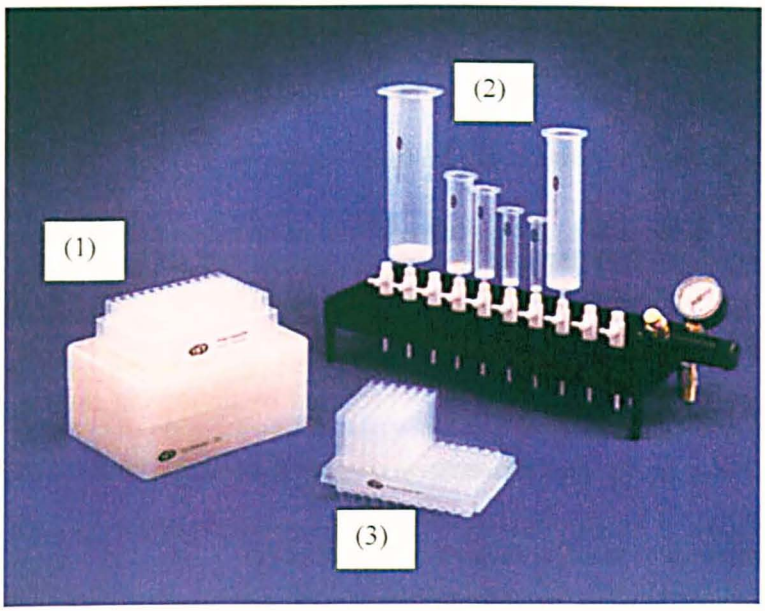

Figure 2-2 Solid phase extraction (SPE) systems

(1): 96- well plate

(2): Cartridges

(3): Micro plates

Other instruments included water purification system ELGA (PureLab UHQ, United Kingdom), pH meter (Eutech Instruments, Malaysia), filtration system for solvent (Sartorius, Germany), vortex mixer (VELP Scientifica, Italy), MicroCL 21R Centrifuge (Thermo Scientific, Germany), ultrasonic bath (Advantage- Lab, Germany).

2.4.4.1.3 **Reagents and Solutions**

All reagents and solvents used were of analytical grade. Potassium dihydrogenphosphate (KH_2PO_4), dipotassium hydrogenphosphate (K_2HPO_4), sodium hydroxide (NaOH), formic acid (HCOOH), acetic acid (CH_3COOH), phosphoric acid (H3PO4), acetonitrile (ACN) and methanol (MeOH) were purchased from Merck,

KGaA, Darmstadt, Germany. Water was purified using a Purelab UHQ system (ELGA, Marlow, United Kingdom). Cephadroxil, cephalexin, cefaclor, cefixime, amoxicillin and cefuroxime were purchased from Sigma-Aldrich Singapore.

2.4.4.1.4 **Preparation of standards**

2.4.4.1.4.1 Stock solutions

Stock solutions (SS) of six antibiotics were prepared by dissolving the standards in suitable solutions (depending on each antibiotic's solubility) indicated in Table 2-7:

Table 2-7 Preparation of stock solutions of 6 antibiotics

Antibiotic	Concentration (μg/mL)	Solvent
Cefadroxil	10000	HPLC water
Cephalexin	10000	HPLC water
Cefaclor	5000	HPLC water
Cefixime	2000	Water - MeOH (50:50)
Amoxicillin	2000	Methanol
Cefuroxime	2000	Methanol

All stock solutions were stored in fridge at – 20 °C

2.4.4.1.4.2 Blank urines

For quality control and bioanalytical test validation, six anonymised urine samples from healthy children, who did not take any medication within two weeks prior to sampling, were used.

2.4.4.1.4.3 Calibration and quality control solutions

Cephalosporin stock solutions were further diluted with water to obtain fresh working solutions, which were used to prepare the Calibration Curve (CC) and Quality Controls (QC) urine samples subsequently with different concentrations. For

amoxicillin and cefuroxime, calibration curves consist of eight concentrations: 0, 0.2, 0.5, 1, 2, 5, 10, and 20μg/mL. Three quality control spiked urines were made at three concentrations of 2, 4, and 16μg/mL. Similarly, for cephalexin, cefadroxil, cefaclor, and cefixime, calibration curves were prepared at eight concentrations: 0, 0.3, 0.6, 2, 6, 12, 20, and 30μg/mL. Three quality control spiked urines were made at concentrations of 0.5, 10, and 25 μg/mL.

2.4.4.1.5 **Clinical sample preparation and SPE process**

Aliquots of thawed urine samples were centrifuged at maximum speed (13000rpm) for 5 min in an Eppendorf benchtop centrifuge and diluted 5-10 times with water. 250 μL of solutions were subsequently added to 250 μL Formic acid in a 1.5 mL Eppendorf snapcap tube. After being vortexed for about 15 seconds, the mixture was rested on a bench for 2 minutes before being loaded into appropriate 96 well plate as in Table 2-8 and Table 2-9:

Table 2-8 SPE process and parameters for Amoxicillin and Cefuroxime

SPE steps	Solutions	Volume (ml)	Flow rate (ml/min)	EQ time (min)	Vacuum pressure (BAR)
Condition	Solvent (a)	1	2	-	-
	Solvent (b)	1	1	-	-
Load	Sample	0.5	0.5	0.5	-
Wash	Solvent (c)	0.5	1	-	-0.08
Elution	Solvent (d)	0.25x2	1	2.00	-0.05

Solvent (a): Methanol

Solvent (b): Formic acid 2%

Solvent (c): HPLC Water

Solvent (d): Potassium dihyrophosphate 20mM pH 3.0-Methanol [50:50]

Table 2-9 SPE process and parameters for Cephalexin, Cefadroxil, Cefaclor and Cefixime

SPE steps	Solutions	Volume (mL)	Flow rate (mL/min)	EQ time (min)	Vacuum pressure (bar)
Condition	SPE solvent A	1	1-2	-	-
Equilibrate	SPE solvent B	1	1-2	-	-
Load	Pre-treatment sample	0.5	0.5	0.5	-0.07
Wash	SPE solvent B x 2 times	0.5	1	0.25	-0.07
	SPE solvent A	0.5	1	0.25	-0.07
Elute	SPE- solvent C	0.5	0.5	0.3	-0.07

Solvent A: Methanol

Solvent B: Formic acid 2% (v/v)

Solvent C: Di-Potassium Hydrogen phosphate 150M-MeOH [50:50]

As a result of SPE process, undesired components and impurities were eliminated and analytes of interest would be retained for the HPLC process.

2.4.4.2 HPLC parameters

2.4.4.2.1 Chromatography

2.4.4.2.1.1 Chromatographic separation for amoxicillin and cefuroxime

The chromatographic separation was performed at ambient temperature (30℃), using an analytic column LiChroCART 125-4.6 mm, Purospher STAR R.P-8 end-capped (5μm) connected to a guard column LiChroCART 4-4mm, Purospher Star RP-8 end-capped (5 μm). The mobile phase was obtained by mixture of Acetonitrile and 20mM KH_2PO_4 buffer, pH 3.0 by phosphoric acid 50% . The mobile phase was pumped at a flow rate of 1.0 mL/min with an injection volume of 20 μL by an automatic sample injector. DAD was used as a detector which was set at the detection wavelength of

200-400 nm. Gradient elution was used with the ratio of solvent in the two channels as follows:

Detection wavelength condition: Amoxicillin (229nm), Cefuroxime (273nm)

Run time: 17 minutes

Retention times: Amoxicillin # 3.68 min., Cefuroxime # 10.11min.

Table 2-10 Chromatographic separation for amoxicillin and cefuroxime

Time (min)	Flow rate (mL/min)	Mobile phase	
		Pump A (%ACN)	Pump B (% KH2PO4 0.02M pH 3.0)
0	1	5%	95%
3	1	16%	84%
10	1	5%	95%
17	1	5%	95%

2.4.4.2.1.2 Chromatographic separation for Cephalexin, Cefadroxil, Cefaclor and Cefixime

The chromatographic separation was performed at 40°C, using an analytic column LichroCART 250-4.6 mm, Purospher STAR R.P-18 end-capped (5μm) connected to a guard column LiChroCART 4-4mm, Purospher Star RP-18 end-capped (5 μm). The mobile phase was obtained by mixture of Acetonitrile and 20mM KH_2PO_4 buffer, pH 2.8 by phosphoric acid 50%. The mobile phase was pumped at a flow rate varying from 1.2 – 1.4 mL/min with an injection volume of 20 μL by an automatic sample injector. DAD was used as a detector which was set at the detection wavelength of 200-400 nm. Gradient elution was used with the ratio of solvent in the two channels as follows:

Table 2-11 Chromatographic separation for Cephalexin, Cefadroxil, Cefaclor and Cefixime

Time (min)	Flow rate (mL/min)	Mobile phase	
		Pump A (%ACN)	Pump B (% buffer KH2PO4 pH 2,8)
0	1,2	10	90
6,0	1,2	10	90
8,0	1,4	14	86
10,5	1,4	14	86
10,6	1,4	25	75
14,0	1,4	25	75
14,1	1,4	10	90
15,0	1,4	10	90
17,0	1,2	10	90
18	1,2	10	90

Detection wavelength: 265 nm

Run times: 18 minutes

Retention time: Cefadroxil : # 4.1 min

Cefaclor: # 6.9 min

Cephalexin: # 10.8 min

Cefixime # 12.2 min

2.4.4.2.2 **Bioanalytical method validation**

A partial method validation of six antibiotics in children's urine was followed in compliance with the Food and Drug Administration (FDA) bioanalytical method validation guideline [224]. The sensitivity was defined by the limit of detection (LOD = 3 times the signal/noise ratio) and the limit of quantification (LOQ = 3 times the LOD). The lowest calibration standard or lowest limit of quantification (LLOQ) was defined as the concentration for which the analytical response was found to be identifiable and reproducible with a precision (%RSD) not greater than 20%. The

upper limit of quantification (ULOQ) was set at 100 fold the LLOQ. The specificity was performed at the LLOQ and tested the ability of the method to differentiate the analytes (six antibiotics) towards endogenous urines interferences from 6 different donors.

The linearity of calibration was determined in the range of 0.3–30 µg/mL (4 antibiotics: cephadroxil, cefaclor, cephalexin, cefixim) and 0.2-20 µg/mL (amoxicillin, cefuroxim). The calibration curve was determined by least-squares regression analysis plotting of peak-area of antibiotics versus the antibiotic concentrations. The targeted correlation coefficient was $r^2 > 0.99$ for all the calibration curves. Back calculations were made to determine the concentrations of drugs in the QC validation sets and clinical samples. The recovery yields for antibiotics were calculated at QCL, QCM and QCH by comparing the area response of spiked urine samples to that of unprocessed aqueous solutions. Intra-day accuracy and precision were determined using 5 different replicates of QCL, QCM and QCH analysed within the same day. Inter-day accuracy and precision were estimated by analysing 5 replicates of QC sets on 4 consecutive days by different analysts. Precision (%) was expressed as the mean relative standard deviation of the peak areas. Accuracy (%) was calculated as [estimated concentration/nominal value] x 100. The variation of the back calculated concentrations from the nominal concentrations should not vary more than 15 % for precision and range from 85 to 115 % for accuracy [225].

2.4.4.2.3 **Quality control (QC)**

For each assay run on the 96-well microplate, a standard calibration curve and a set of QC samples were also analysed simultaneously. The number of QC samples made up

5% of 96 wells in the microplate and were set at 3 concentrations low, medium and high among which one QC sample was placed to be the first to be analysed, another was the last and the rests were placed randomly in the microplate.

The results of the QCs provided the basis for accepting or rejecting the batch of clinical samples. The acceptance criteria for the QCs are < 15% in precision (% relative standard deviations - RSD) and between 85 and 115 % for accuracy (deviation from nominal value). No more than two QCs may be outside the accuracy range, but not at the same level. The batch of samples is rejected and must be reanalysed if the QCs exceed the acceptance criteria limits.

2.4.4.3 HPLC process for patients' samples

To start with, the HPLC system was equilibrated and a system suitability test was performed using a suitable working solution. Then, 20 µL of each sample was injected into the equilibrated HPLC system using the auto sampler sequence. The retention times for each peak in the clinical sample were acceptable if they diverged less than 5% from the same peak in a calibrator in the same concentration range and the spectra for peaks in the samples that DAD obtained must follow the same pattern between 200-400 nm as a calibrator in the same concentration range.

Parameters which were used for result interpretation included: retention time, peak area, the purity of the peak and similarity between UV spectrums of analytes in the sample and in the standard solutions.

2.4.4.4 Interpretation of results

The presence of an antibiotic in a sample would be determined if the following criteria were met:

- The retention time of antibiotic in the sample was similar to that of the control.
- Peak area in the sample was equal to or bigger than that at the lowest concentration in the standard calibration curve.
- The purity of the peak in the sample was equal to that at the lowest concentration in the standard calibration curve.
- The similarity between UV spectrums of the analyte in the sample and in the standard solutions was greater than 90%.

Chapter 3

Presentation and Treatment Characteristics of Outpatients with Acute Respiratory Infections in Children's Hospital 1

3.1 Introduction

ARI is the most frequent disease among children world-wide, with higher morbidity and mortality rates in developing countries. In Vietnam, ARI is the most common reason for medical consultation among children in outpatient settings. Here, we aim to describe the demographic, clinical and epidemiological characteristics of children 15 years of age or younger with mild ARI in Vietnam, and review clinical management (in particular, antibiotic use) at the outpatient clinic of CH1 during a one-year period from 2009 to 2010. CH1 is one of the two major children's hospitals serving Ho Chi Minh City.

3.2 Materials and methods

The data for this study are derived from the prospective descriptive study (03AV) conducted in the outpatient department of CH1 from the beginning of February 2009 to the end of January 2010. Details of the study's design, materials and methods are described in Chapter 2.

Patients visiting the CH1 outpatient clinic are first seen at the registration and triage desks, where nurses register and classify patients into the appropriate specialties based on their chief complaints. There are written standard operating procedures (SOP) at the outpatient clinic for how children should be triaged. In this way, children with ARI symptoms are sent to either the general examination rooms (n = 20) or the respiratory examination rooms (n = 2). However, because these respiratory

examination rooms are designated for the severe end of outpatient respiratory diseases, requiring consultation from respiratory experts, children with LRI or with severe or prolonged respiratory symptoms are more likely to be sent to these two respiratory examination rooms than other examination rooms. Patients with mild URI as rhinitis, laryngitis and ear or sinus problems are usually seen in the dedicated ear-nose-throat examination rooms.

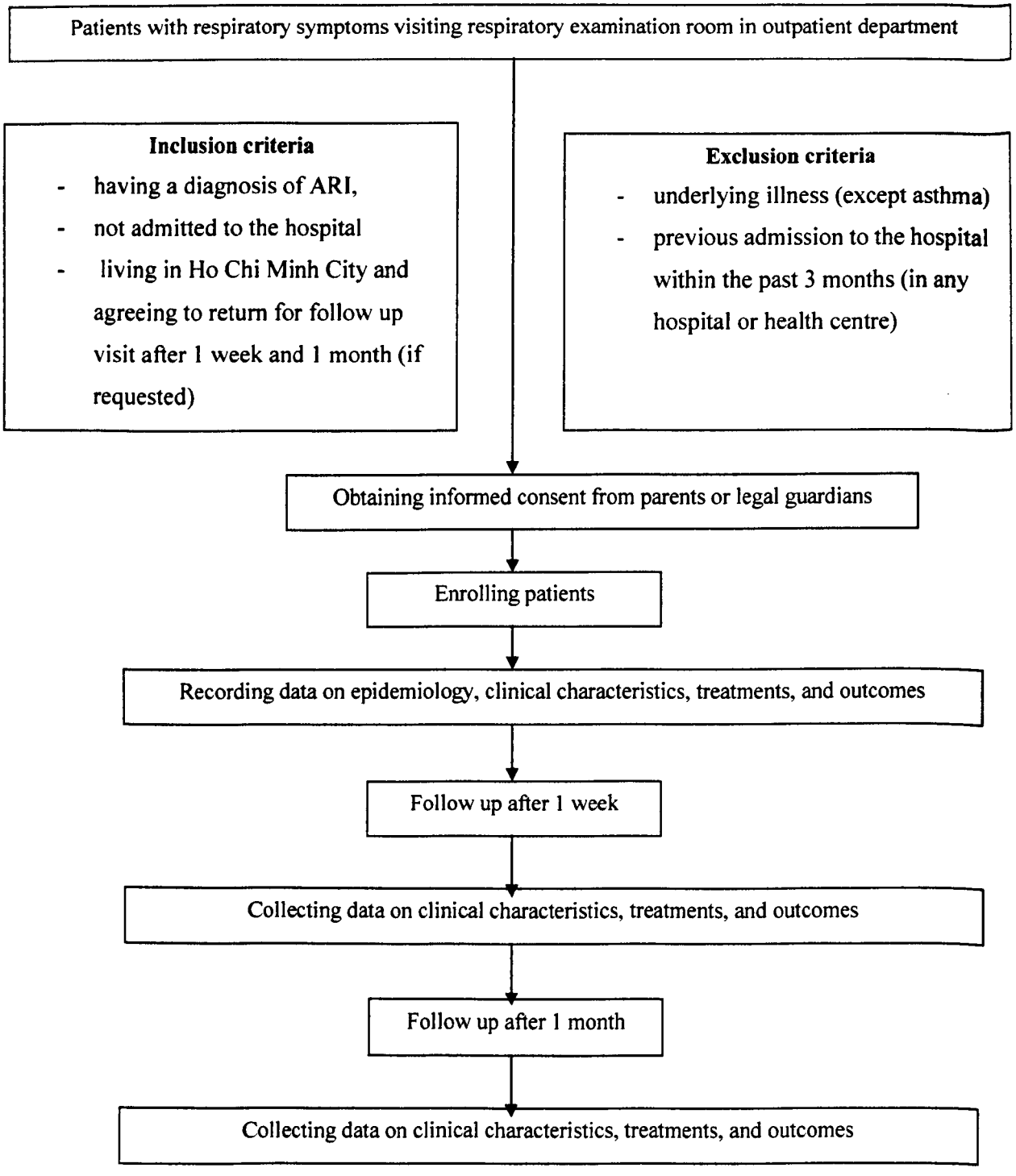

Figure 3-1 Study flow diagram

3.3 Results

3.3.1 Demographic Characteristics

Over a period of one year from 9th February 2009 to 6th February 2010, there were 870,000 patients with ARI visiting the outpatient department at CH1 and 45,000 children coming to the respiratory examination rooms (annual statistical data from CH1). On average, there were about 120 patients per day being examined at the two respiratory rooms where our study was conducted.

Patient enrolment for this study was performed every week from Monday to Thursday during specified timeslots (Monday 8-9am, Tuesday 9-10am, Wednesday 10-11am, Thursday 1-2pm) until a maximum of four patients per day or twelve per week were enrolled in the study.

In total, 563 (0.064% of the total of patients with ARI) patients were enrolled in this study during the one-year period.

Enrolled patients had a median age of 1.96 years (inter-quartile range [IQR]: 1.05-3.18) and 95.2% of patients were aged 5 years or below. The male: female ratio was 1.28:1. All 563 patients were from the 22 districts in Ho Chi Minh City (as this was an inclusion criterion of the study), the majority of whom were from districts directly surrounding CH1 (District 10), such as Binh Tan, Binh Chanh, Tan Binh, and Tan Phu (Table 3-1and Figure 3-2).

Table 3-1 Distribution of places of residence of patients by district

District	Number of patients	Percentage (%)
Binh Tan	86	15.3
Binh Chanh	76	13.5
Tan Binh	57	10.1
Tan Phu	57	10.1
Hoc Mon	46	8.2
8	42	7.5
12	37	6.6
10	35	6.2
11	27	4.8
6	26	4.6
Cu Chi	13	2.3
5	13	2.3
7	11	2.0
Go Vap	9	1.6
3	8	1.4
Thu Duc	6	1.1
9	6	1.1
4	4	0.7
Binh Thanh	2	0.4
Nha Be	1	0.2
Phu Nhuan	1	0.2
Total	**563**	**100**

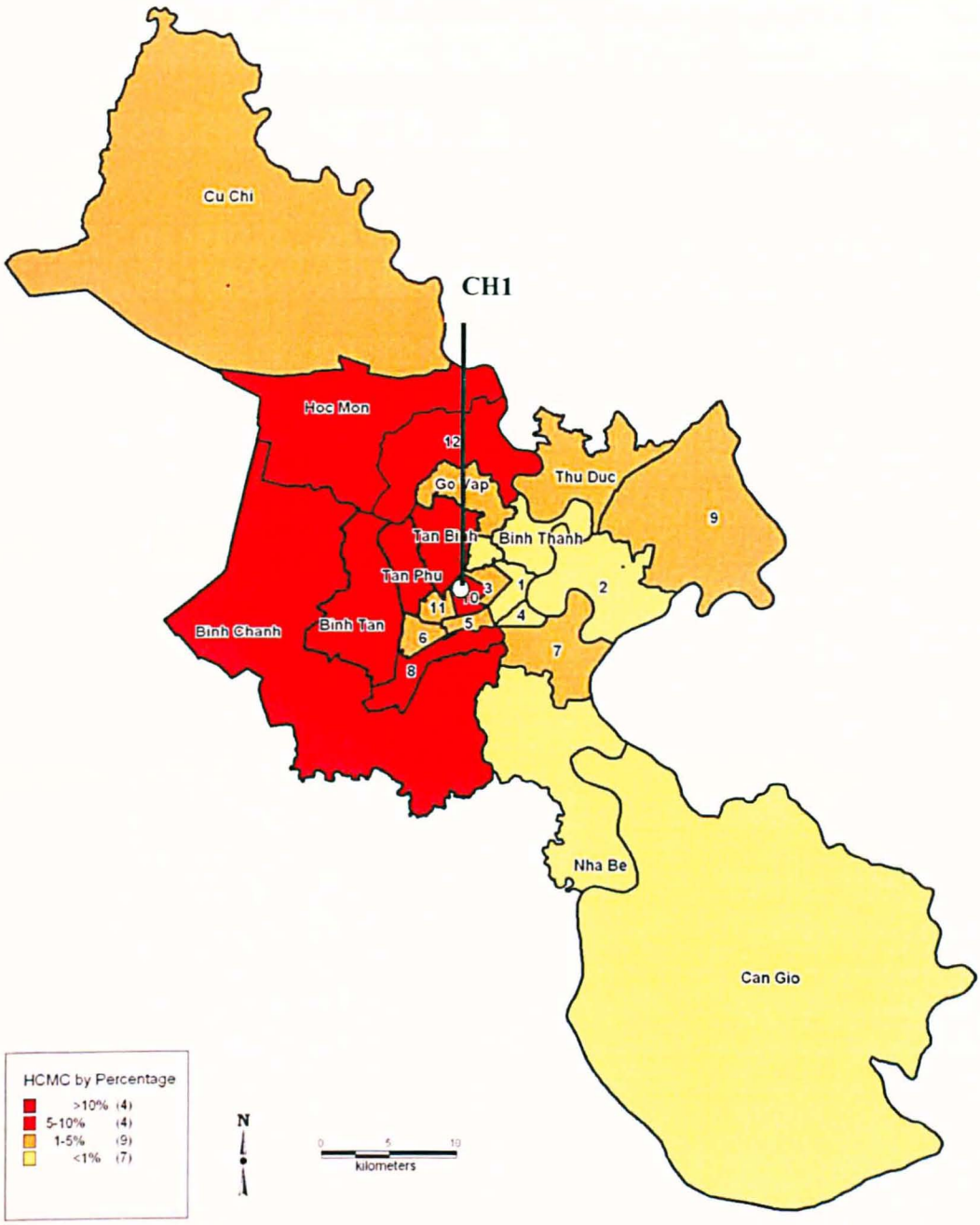

Figure 3-2 Distribution of places of residence of patients by district

3.3.2 Clinical characteristics of outpatients with ARI

Table 3-2 Epidemiology & clinical characteristics by age

	≤ 1 year n =137	1-≤ 2 years n= 148	2- ≤ 5 years n= 251	>5 years n= 27	Total N=563
Male, n(%)	80 (58.4)	85 (57.4)	137 (54.6)	14 (51.9)	316 (56.1)
Median age in years (IQR)					1.96 (1.05-3.18)
Respiratory symptoms					
Cough	136 (99.3)	147 (99.3)	250 (99.6)	26 (96.3)	559 (99.3)
Productive cough	114 (83.8)	124 (83.5)	200 (79.5)	16 (59.1)	454 (80.6)
Sore throat	1 (0.7)	3 (2.0)	56 (22.3)	12 (44.4)	72 (12.8)
Runny nose	115 (83.9)	128 (86.5)	195 (77.7)	16 (59.3)	454 (80.6)
Nasal congestion	100 (73.0)	95 (64.2)	146 (58.2)	12 (44.4)	353 (62.7)
Other symptoms					
Conjunctivitis	7 (5.1)	3 (2.0)	6 (2.4)	0(0)	16 (2.8)
Shortness of breath	26 (19.0)	16 (10.8)	19 (7.6)	3 (11.1)	64 (11.4)
Cyanosis	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Diarrhoea	12 (8.8)	6 (4.1)	7 (2.8)	0 (0)	25 (4.4)
Rash	4 (2.9)	4 (2.7)	3 (1.2)	0 (0)	11 (2.0)
Examination on presentation					
Median pulse (IQR)	119 (110-120)	117 (110-120)	112 (100–118)	100 (98-110)	116 (100-120)
Median temperature (IQR)	36.8 °C (36.4-37.0)	36.8 °C (36.3-37.0)	36.8 °C (36.3-37.1)	36.7 °C (36.5-37.0)	36.8 °C (36.3-37.0)
Fever, n (%)	16 (11.8)	18 (12.4)	39 (15.5)	3 (11.1)	76 (13.5)
Median Breath rate (IQR)	40 (35-41)	36 (32-40)	32 (30-36)	30 (29-36)	35 (30-40)
Fast breathing	1 (0.7)	18 (12.2)	14 (5.6)	0 (0)	33 (5.9)
Heart sound normal, n(%)	137 (100)	148 (100)	251 (100)	27/27(100)	563 (100)
Chest in-drawing, n (%)	0(0)	2 (1.4)	3 (1.2)	0 (0)	5 (0.9)
Rhonchi, n (%)	110 (80.3)	105 (70.9)	137 (54.5)	10 (37.0)	362 (64.3)
Stridor, n (%)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)
Crackles, n (%)	50 (36.5)	47 (31.8)	54 (21.5)	1 (3.7)	152 (27.0)
Treatment prescribed on presentation					
Antibiotics prescribed	137 (100)	146 (98.6)	251 (100)	27 (100)	561 (99.6)
Other treatments prescribed	137 (100)	148 (100)	250 (99.6)	27 (100)	562 (99.8)
Bronchodilator	94 (68.6)	85 (57.4)	134 (53.4)	11 (40.7)	324 (57.6)
Corticosteroids	11 (8.0)	16 (10.8)	28 (11.2)	3 (11.1)	58 (10.3)
Mucolytic agent	16 (11.7)	12 (8.1)	27 (10.8)	7 (25.9)	62 (11)
Cough syrup	97 (70.8)	108 (73)	171 (68.1)	18 (66.7)	394 (70)
Antipyretics	28 (20.4)	39 (26.4)	76 (30.3)	7 (25.9)	150 (26.6)
Antihistamine	14 (10.2)	21 (14.2)	25 (10.0)	6 (22.2)	66 (11.7)

	≤ 1 year n =137	1-≤ 2 years n= 148	2- ≤ 5 years n= 251	>5 years n= 27	Total N=563
Epidemiological & medical history					
History of asthma, n(%)	1 (0.7)	3 (2.0)	11 (4.4)	5 (18.5)	20 (3.6)
Current use of antibiotics, n(%)					
Yes	26 (19.0)	37 (25.0)	54 (21.5)	8 (29.6)	125 (22.2)
Unknown	48 (35.0)	63 (42.6)	107 (42.6)	13 (48.1)	231 (41.0)
Use antibiotic for current chief complaints	24/26 (92.3)	35/37 (94.6)	54/54 (100)	8/8 (100)	121/125 (95.3)
Last month use of antibiotics, n(%)					
Yes	23 (16.8)	21 (14.2)	46 (18.3)	8 (29.6)	98 (17.4)
Unknown	44 (32.1)	56 (37.8)	75 (29.9)	4 (14.8)	179 (31.8)
Median number of household members (IQR)	4 (4-6)	4 (4-6)	4 (4-6)	4 (4-5)	4 (4-6)
Median number of household members under 5 years old (IQR)	1 (1-2)	1 (1-1.75)	1 (1-2)	0	1.2 (0.6-1.7)
Median number of rooms in house	2 (1-2)	2 (1-2)	2 (1-2)	2 (1-2.3)	2 (1-2)
Previous hospital admissions for respiratory problems, n (%)	9 (6.6)	19 (12.8)	47 (18.7)	5 (18.5)	80 (14.2)
< 3 times of admissions	9/9 (100)	19/19 (100)	47/47 (100)	5/5 (100)	74/80 (92.5)
Previous hospital admissions for other reasons, n (%)	10 (7.3)	25 (16.9)	49 (19.5)	5 (18.5)	89 (15.8)
Other household members ill during the past 3 weeks, n(%)	48 (35.0)	47 (31.8)	77 (30.7)	10 (37.0)	182 (32.3)
Any classmates or play mates ill during the past 3 weeks, n(%)					
Yes	9 (6.6)	7 (4.7)	19 (7.6)	2 (7.4)	37 (6.6)
Unknown	77 (56.2)	99 (66.9)	163 (64.9)	16 (59.3)	355 (63.1)
Diagnosis (1st follow up)					
Bronchitis	31 (22.6)	72 (48.6)	151 (60.2)	8 (29.6)	262 (46.5)
Bronchiolitis	82 (59.9)	34 (23.0)	6 (2.4)	0	122 (21.7)
Rhino - pharyngitis	17 (12.4)	31 (20.9)	47 (18.7)	10 (37.0)	105 (18.7)
Pharyngitis	6 (4.4)	6 (4.1)	23 (9.2)	7 (25.9)	42 (7.5)
Asthma	1 (0.7)	3 (2.0)	11 (4.4)	2 (7.4)	17 (3.0)
Pneumonia	0	2 (1.4)	5 (2.0)	0	7 (1.2)

	≤ 1 year n =137	1-≤ 2 years n= 148	2-≤ 5 years n= 251	>5 years n= 27	Total N=563
Tonsillitis	0	0	7 (2.8)	0	7 (1.2)
Laryngotracheobronchitis	0	0	1 (0.4)	0	1 (0.2)
Outcome (after 1 week)					
Complete recovery	32 (23.4)	31 (20.9)	68 (27.1)	7 (25.9)	138 (24.5)
Partial recovery	89 (65.0)	97 (65.5)	158 (62.9)	17 (63.0)	361 (64.1)
Unchanged	11 (8.0)	12 (8.1)	10 (4.0)	3 (11.1)	36 (6.4)
Worsened	2 (1.5)	6 (4.1)	8 (3.2)	0	16 (2.8)
Admitted to hospital	2 (1.5)	0	5 (2.0)	0	7 (1.2)
Unknown	1 (0.7)	2 (1.4)	2 (0.8)	0	5 (0.9)

Table 3-2 presents the epidemiological and clinical characteristics of study participants by four age groups: under 1 year, 1-2 years, 2-5 years and over 5 years old. While there was no difference regarding the gender of patients, a significant difference was found in the number of patients in 4 age groups (Kruskal Wallis test, p value < 0.001). There was a relative over-representation of children between 2 and 5 years old, and only a few ARI patients were older than 5 years.

To be eligible for the study, patients had to have at least one of four respiratory symptoms as the chief complaint: cough, running nose, nasal congestion or sore throat. Cough was the most commonly reported complaint, present in 559/563 (99.3%) patients. Productive cough was recorded in 454/563 (80.6%) patients and the proportion of productive cough in children below five years old (81.7%, 438/536) was higher than that in the above five group (59%, 16/27). The second most frequent symptom was runny nose, which was reported in 454/563 (80.6%) patients in the whole study population and in 243/285 (85.2%) patients under the age of two. Almost 63% (353/563) of patients experienced nasal congestion and the proportion of infants

(children less than one year old) (73%, 100/137) exceeded the proportion of patients over 5 years with the same symptom (44%, 12/27).

Around 13% (72/563) of patients reported having sore throat, which was mostly seen in older children (44% of patients over 5 years old). More than 11% (64/563) of patients were reported by their parents as having dyspnoea during the period prior to presentation.

With regard to clinical signs on physical examination, fever, fast breathing and chest in-drawing were recorded in 13.5% (76/563), 5.9% (33/563), and 0.9% (5/563) of patients, respectively. In our study, fever is defined as axillary temperature above 37.5°C while fast breathing is defined by respiratory cut-off rates developed by WHO (50 breaths per minute for 2-12 month infants and 40 breaths/minute for 1-5 year old children) [226]. There were no patients with cyanosis. Findings of lower respiratory tract involvement, as indicated by the presence of rhonchi or crackles, were found in 64.3% (362/563) and 27% (152/563) of patients, respectively, with rhonchi manifesting in approximately 75% (215/284) of patients below 2 years.

Among symptoms outside the respiratory tract, diarrhoea, conjunctivitis and rash were seen in 4.4% (25/563), 2.8% (16/563) and 2.0% (11/563) of patients, respectively, all of whom were under 5 years.

Most of the families in the study were nuclear families (two parents and their children) with the median number of household members in a family at 4 and the mean at 5. The median number of rooms, at 2, indicated that there was one bedroom and one dining room on average in most of the families. About one-third of patients (182/563) reported that other members in their families had symptoms of a respiratory infection within three weeks before their children presented to the hospital.

The proportion of patients who had other household members contracting respiratory infections within the last three weeks in large families (6 members or more) (39%-62/158) was significantly higher than that in small families (less than 6 members) (30% - 120/405) (Chi-square test, p value = 0.02). There was no significant difference in the median number of household members among patients with upper tract respiratory infections (URI) and those with lower tract respiratory infections (LRI) (p value = 0.8).

3.3.3 **Diagnosis**

In Table 3-2 above, diagnoses on the Day 7, which were likely more accurate than those on the Day 0 in our judgement, were presented and used to assess the appropriateness of antibiotic use.

72.6% (409/563) of cases were classified as LRI, of which bronchitis (262/563, 46.5%) was the most frequent diagnosis, followed by bronchiolitis (122/563, 21.7%), asthma exacerbation (17/563, 3.0%), and pneumonia (7/563, 1.2%). The remaining (154/563, 27.4%) were categorised as URI, which consisted of nasopharyngitis (105/563, 18.7%), pharyngitis (42/563, 7.5%), and tonsillitis (7/563, 1.2%).

While URI were seen more commonly in the children aged over 5 years, LRI were observed more frequently in the under 5 year-old group (p value < 0.001). Among LRI, bronchiolitis was mainly found in children less than 2 years old while bronchitis was observed in all age groups.

3.3.4 Treatment of ARI in outpatient department at the time of presentation

561/563 (99.6%) patients were prescribed antibiotics on the day of presentation. Two children who were diagnosed with rhino-pharyngitis received only treatment for symptom relief and no antibiotics.

The median duration of antibiotic prescription was 6 days (IQR: 4-7 days), of which 191/563 (34%) patients were prescribed antibiotics for less than five days and 4.5% (25/563) of patients were prescribed antibiotics for 10 days or more. Of note is the fact that there was one patient who was eventually diagnosed with rhino-pharyngitis but received antibiotic for 19 days. 33.7% (189/561) of patients were prescribed other antibiotics during follow-up visits than the ones prescribed initially. The most commonly used antibiotics prescribed at the time of presentation were amoxicillin-clavulanic acid (45.6%), cefuroxime (22.0%), cefixime (11.4%), cefaclor (8.2%), erythromycin (3.7%), amoxicillin (3.0%), and cefpodoxime (2.3%), while cotrimoxazole accounted for only 0.4%. A similar spectrum of antibiotics was prescribed for both URI and LRI. In patients with URI, amoxicillin-clavulanic acid was the most frequently prescribed antibiotic, at 34.4%, followed by cefuroxime (24%), cefixime (13.6%), and cefaclor (13%). Likewise, in patients with LRI, the four most common antibiotics were amoxicillin-clavulanic acid (49.9%), cefuroxime (21.3%), cefixime (10.5%), and cefaclor (6.4%). Looking at the class of antibiotics, the prescription rate for amoxicillin-clavulanic acid in patients with LRI (49.9%) was higher than that in patients with URI (34.4%) (Chi-square test, p value < 0.001), whereas the use of cephalosporins (cefuroxime, cefixime, cefaclor, cefpodoxime) in URI (51.9%) was higher than that in LRI (40.8%) (Chi-square test, p value < 0.001). The use of first choice antibiotics, as recommended by guidelines, such as amoxicillin

and cotrimoxazole was very low in both URI and LRI, at 5.8% and 4.2%, respectively.

Besides antibiotics, 562/563 (99.8%) patients were prescribed other medications. Seventy percent of patients received cough syrups, and 11% were given mucolytic agents to stimulate mucus expectoration from the respiratory tract. 11% of children were given antihistamines, which suppress symptoms of allergy and hyperreactivity such as running nose or sneezing. 14/137 (10.2%) of children below 1 year old and 13/66 (19.7%) patients with bronchitis and bronchiolitis were prescribed antihistamines, despite the fact that this medication is not recommended for infants and for LRI [99, 227]. 43/66 (65%) patients with productive cough received antihistamines.

323/563 (57.4%) patients were prescribed bronchodilators, of whom 4% (13/323) had asthma, 92% (297/323) had LRI including bronchiolitis or bronchitis or pneumonia, and the remaining 4% (13/323) had URI such as pharyngitis, tonsillitis, and rhino-pharyngitis.

58/563 (10.3%) patients received oral corticosteroids, of whom only 10/58 (17.3%) were diagnosed with asthma. The remaining patients (48/58, 82.7%) had non-asthmatic diagnoses such as pharyngitis, rhino-pharyngitis, bronchitis, and bronchiolitis.

Before coming to the hospital, 125/563 (22.2%) patients had already taken antibiotics within 2 days prior to presentation. In 41% of cases, the parents did not know whether the children received antibiotics or not, as they bought medications from pharmacies or private clinics without prescriptions. The pharmacists or treating doctors in private clinics usually gave patients separate pills/medications without package or inserts.

98/563 (17.4%) patients had used antibiotics within the last one month for another indication than the current illness, of which 68/98 (69.3%) patients had taken antibiotics within the last 7 days. For 179/563 (31.8%) children, the status of antibiotic use was unknown.

3.3.5 **Outcome of outpatients with ARI**

In this study, patient's outcome was classified into seven categories: (1) complete recovery (all symptoms present at first presentation have resolved); (2) partially recovered (one or more symptoms that were present at first presentation are still present, but there are less symptoms and/or they are less severe); (3) unchanged (symptoms present at presentation are still present, with similar severity as on presentation); (4) worsening (symptoms present at first presentations have become more severe, and/or the child experiences additional symptoms related to respiratory illness); (5) admitted to hospital; (6) death; and (7) lost to follow-up.

After 1 week, 499/563 (88.6%) patients had a good outcome and were either fully recovered (138/563, 24.5%) or partially recovered (362/563, 64.1%). Regarding respiratory symptoms, Table 3-3 shows that the proportion of children reporting cough dropped significantly by around 26%, from 99.6% of 563 patients on presentation to about 73.7% on day 7. Likewise, the proportion of nasal congestion, runny nose, sore throat, and fever decreased considerably by 43.9%, 37.2%, 10.8%, and 10.2%, respectively. In terms of lung sounds on auscultation, there was a substantial fall in the rate of rhonchi heard on examination by almost one-third, from 64.3% (362/563) on Day 0 to 34.1%(185/5453) on Day 7, while the proportion of crackles declined by about 21%.

Table 3-3 Respiratory manifestations on Day 0 and Day 7

Manifestations	Day 0	Day 7
Cough	559 (99.3)	401 (73.7%)
Running nose	454 (80.6)	236 (43.4%)
Nasal congestion	353 (62.7)	102 (18.8%)
Sore throat	72 (12.8)	11 (2%)
Fever	76 (13.5)	18 (3.3%)
Rhonchi	362 (64.3)	185 (34.1%)
Crackles	152 (27.0)	35 (6.4%)

In contrast, 59/563 (10.5%) patients showed poor outcomes: 6.4% remained clinically unchanged, 2.8% worsened and 1.2% were admitted to the hospital.

Among 138 patients with full recovery, 53 (38.4%) were diagnosed with URI, while 23/23 (100%) patients who became more serious or had been hospitalised were eventually diagnosed with LRI such as bronchitis, bronchiolitis, pneumonia, and asthma. Patients with LRI had a higher rate of poor outcome (unchanged or worsened) than those with URI (12.2% vs. 5.8%, Chi-square test, p value = 0.039). Likewise, a higher proportion of full recovery was observed in the URI group than in the LRI group (34.4% vs. 20.7%, Chi-square test, p value = 0.001).

A very low number of patients (18/563, 3.2%) did not return for follow-ups, of which 5 patients' parents could not be reached by telephone. The parents of the remaining 13 patients were contacted by telephone: two children became worse and went to other clinics and 11 patients recovered completely and their parents were too busy to bring them for follow-up.

3.4 Discussion

This is the first study conducted in (southern) Vietnam to describe the epidemiology, clinical characteristics and treatment of outpatients presenting with ARI. Almost 95% of patients in this study were below 5 years and more than 50% were under the age of two, which is consistent with outpatient studies from other countries [228-231]. The male: female ratio in our study was 1.28:1 which is also similar to these other studies [228-230].

Regarding household size, 28% (158/563) of patients in our study lived together with five or more people in the same house. There was no significant difference in the median number of household members among patients with URI and those with LRI. In a study from Egypt, the proportion of severe disease (pneumonia or other) increased considerably with larger family size [230].

The proportion of patients who had other household members contracting respiratory infections within the last three weeks in large families (6 members or more) (41%) was significantly higher than that in small families (less than 6 members) (30%) (Chi-square test, p value = 0.02). This may suggest that overcrowding in houses increases the probability of contracting ARI from household members as it promotes fomite and airborne transmission of respiratory pathogens. A similar finding was seen in a study in outpatients in Tikrit General Teaching Hospital, Iraq, which showed that children living in houses with crowding index of more than 5 were more likely to contract ARI than those living in houses with crowding index equal to or less than 5 [232]. However, in a prospective cohort study carried out in Turkey on 204 infants evaluated by home visits, there was no association between the number of persons per room and ARI incidence [233].

Cough was the most commonly reported symptom, by almost 99% (554/563) of all patients, followed by running nose (80.6%) and nasal congestion (62.7%). Fever was seen in 13.5% (76/563) of cases. Around 13% of patients reported sore throat, which was mostly seen in older children (44.4% of patients over 5 years old), presumably because younger children (less than 2 years old) were unable to communicate that they had a sore throat. Regarding physical examination signs, fast breathing was observed in 5.9% of patients and chest indrawing in 0.9%. Among abnormal sounds on lung auscultation, rhonchi and crackles were the most frequent signs, accounting for 64.3% (362/563) and 27% (152/563), respectively.

Similar findings were observed in a community-based study conducted between 1985 and 1987 in Nigeria, where cough was also the most common symptom accounting for 87% to 96% of under five year old children with ARI. Nasal discharge was also the second most frequent symptom, from 67% to 83% [231]. However, the rates of fever (60% to 85), rapid breathing (7.4 to 17.2%) and chest retraction (2% to 17%) in the Nigerian study were significantly higher than in ours. This may be due to the fact that the authors of the Nigerian study were seeing a group of children with more severe ARI than in our study. In addition, for the symptom of fever, it was recorded in our study just at the time when patients presented on Day 0 while many patients may have had high temperature in previous days before presentation.

With regard to diagnosis, LRI outnumbered URI by almost 3 times in our study. However, data from all ARI cases presenting to the outpatient clinic suggest that URI (61%) were observed much more commonly than LRI (39%). The higher proportion of LRI enrolled in our study likely reflects the fact that it was conducted in respiratory examination rooms rather than in general examination rooms, and these

rooms are designated for more severe cases requiring consultations from respiratory experts. As a result, the number of LRI, such as bronchitis, bronchiolitis, or pneumonia in respiratory rooms and in our study is higher than that in the whole outpatient department. Nearly half (46.5%) of the enrolled patients were diagnosed with bronchitis, and bronchiolitis and rhino-pharyngitis were the second and third most common diagnoses, accounting for 21.7% and 18.7% of all diagnoses, respectively. There was no case with otitis or sinusitis. Statistical data from Children's Hospital 1 in the same year of 2009, when our study was conducted, showed that among patients with ARI in the whole outpatient clinic, pharyngitis was the most common diagnosis, at around 33%, followed by nasopharyngitis (24.2%), bronchitis (20.6%), and bronchiolitis (11.6%). Meanwhile, patients with otitis media and sinusitis are referred to otorhinolaryngology rooms. There was no correlation between gender and URI or LRI in our study whereas a significant difference was found between age and URI or LRI (p value < 0.001). The younger patients were more likely to contract LRI; 83% of under 1 year old patients were diagnosed with LRI while among patients over 5 years of age, only 37% had LRI. The study in Tikrit General Teaching Hospital in Iraq also showed a high correlation between age and ARI severity which was categorised as no pneumonia, pneumonia, severe pneumonia, and very severe disease [232].

Regarding the ambulatory treatment of ARI, as in many other countries, physicians in Vietnam and CH1 use the WHO's IMCI guideline and also the National Therapeutic Guideline established by the Ministry of Health of Vietnam. According to the IMCI guidelines (Appendix F), antibiotics are indicated only in individuals with pneumonia, severe pneumonia or otherwise very severe disease, and ear infections. For children

with only a cough or a common cold (no pneumonia), no antibiotics are recommended but only symptom relief measures such as antipyretics to reduce high fever or cough syrup to soothe the throat and alleviate the cough [12]. Similarly, in the National Therapeutic Guidelines from the Ministry of Health of Vietnam (Appendix H), pneumonia and streptococcal pharyngitis are the two main indications for antibiotics. For other ARIs such as nasopharyngitis, rhinitis, bronchitis or bronchiolitis, antibiotics have not proven effective and therefore are not recommended unless there are bacterial super-infections [234]. According to these two guidelines, amoxicillin or cotrimoxazole are the drugs of choice for ambulatory treatment of pneumonia in children while amoxicillin, penicillin or erythromycin is recommended for streptococcal pharyngitis. The 2011 Nelson's textbook of paediatrics and the most recent evidence-based guidelines and Cochrane reviews include similar recommendations in terms of indications and choice of antibiotics for ARI in children. In brief, there is no evidence to show that antibiotics are effective in most ARI in children, apart from pneumonia and bacterial pharyngitis [99-102, 235, 236].

In our study, a very high number (561/563-99.6%) of ARI outpatients were prescribed antibiotics. This figure clearly indicates indiscriminate antibiotic use in CH1 although CH1 has issued and distributed its own ARI guidelines (Appendix G), which point out the indications for antibiotic use in ARI, limited to pneumonia, GAS pharyngitis or otitis media, to every physician in the hospital.

Antibiotics are not indicated for acute URI except for group A beta-haemolytic streptococci (GAS, *S. pyogenes* and others) to prevent acute rheumatic fever [99, 100, 236] which is a complication of about 3% of cases of streptococcal pharyngitis and the very rare URI caused by *Corynebacterium diphtheriae* and *Neisseria gonorrhoea*.

GAS pharyngitis is a common form of (bacterial) pharyngitis, responsible for about 20 – 30% of sore throat visits in children [237], and occurs predominantly in children aged 5 to 15 years. The prevalence of GAS pharyngitis is significantly lower in the under 3 year-old group [236]. In our study, all except one of 154 patients with URI were treated with antibiotics, and 55.8% (86/154) were under the age of 3. Of the remaining 68 patients aged 3 years or more, 100% were given antibiotics, none of whom were diagnosed with GAS pharyngitis. This is due to the fact that physicians at CH1 cannot differentiate GAS pharyngitis from viral pharyngitis based on clinical judgement during presentation alone, as rapid antigen detection tests (RADT), which are recommended for diagnosis of GAS pharyngitis in children over 3 years old [236], have not been adopted in most outpatient clinics in Vietnam, including CH1. Therefore, physicians choose to treat all treatable diagnoses, including GAS pharyngitis, even in children younger than 3. With respect to clinical assessment, although all physicians in CH1 are aware of clinical scoring systems for diagnosis of GAS pharyngitis, they have not applied these, mostly because of time constraints. Moreover, most of the children with ARI in this study were under 5 years old, while GAS pharyngitis is seen mostly in patients over 5.

In the WHO guidelines for IMCI, antibiotics were indicated for patients with signs of pneumonia (high respiratory rate, corrected for age) or very severe disease (chest indrawings or inspiratory stridor) [12]. In addition, antibiotics are not routinely indicated for 2 – 59 month-old children with non-severe pneumonia with a wheeze but no fever, according to recent evidence-based recommendations from WHO [238]. However, in our study, fast breathing was observed in only 5.9%, chest indrawing in

0.9%, and pneumonia was clinically diagnosed in only 1.2% of patients while 99.6% of children received antibiotics.

For bronchitis and bronchiolitis, antibiotics are not recommended for outpatients unless they have complications, such as secondary bacterial pneumonia or respiratory failure, and are hospitalised. [99, 101, 235, 239]. Moreover, unnecessary antibiotic prescriptions may bring about adverse effects such as allergy, anaphylactic reactions, gastro-intestinal disorders and particularly lead to increases in antibiotic resistance.

Table 3-4 Studies on antibiotic use in children with ARI

Author; Year; Country	Method	Participants	Study settings	Study period	Results	
					Rate of antibiotic use	Most commonly used antibiotics
Nguyen QH; 2011, Vietnam[96]	Questionnaire survey	children under five with mild ARI	Community, private clinic and public clinic	2007	80% of children with mild ARIs coming to public clinics	In mild ARI: betalactam penicillin (58%), cephalosporins (27%), sulphonamides and trimethoprim (7%), macrolides (9%) and other antibacterials (3%).
Tabatabaei S.A; 2007, Iran [106]	Prospective cross-sectional study	Children aged between one month and 15 years with acute respiratory symptoms	Outpatient clinics	2007-2008	33%	Amoxicillin-clavulanic acid (34.5%), amoxicillin (20.8%), azithromycin (17.5%), erythromycin (8.7%), and penicillin (8.5%).
Thamer.K.Yousif Et al; 2006, Iraq [232]	Hospital based longitudinal study	children under 5 years old with ARIs	outpatient department in teaching hospital	2004-2005	83.4% (pneumonia); 56.4% (non-pneumonic ARIs)	–
Farideh Shiva; 2006, Iran [103]	cross-sectional study	Outpatient children with acute infections including respiratory infections	three different settings of ambulatory care: private offices, general health facilities, and hospital based clinics	2003	87.8% by general practitioner; 83.9% by paediatricians	–
Annemiek E. Akkerman et al;	Observational study in general practitioners from the	0–15 year old children with respiratory	general practitioners' clinics	2000	25%	–

2004, the Netherland [107]	Integrated Primary Care Information (IPCI) database.	infections,				
Michael A. Steinman; 2003, the United States [104]	Cross-sectional survey	Patients (adult and children) visiting community-based outpatient clinics	outpatient clinics	three 2-year periods (1991–1992, 1994–1995, and 1998–1999)	For nonpneumonic acute respiratory tract infections: 75% in 1994–1995 and 80% in 1998–1999	–
Mattias Larsson; 2000, Vietnam [95]	community-based survey	children between 1 and 5 years of age with respiratory symptoms	community	1999	91%	ampicillin (74%), penicillin (12%), amoxicillin (11%), erythromycin (5%), tetracycline (4%) and streptomycin (2%)
Ann-Christine Nyquist; 1998, the United States [240]	national survey of practicing physicians	children younger than 18 years with diagnosis of cold, upper respiratory tract infection (URI), or bronchitis	Office-based physician practices	1992	44% in common colds, 46% in URIs, and 75% in bronchitis	–

The high rate of antibiotic use in our study is in line with a study in 1999 in Ba Vi district, Ha Tay province, Vietnam, which involved a standardised questionnaire survey of 166 children's caregivers on antibiotic use for their children with ARI. This study indicated that 91% of children with ARI used antibiotics [95]. Likewise, in another study in Ba Vi, Ha Noi, carried out 8 years later in 2007, among 219 children with mild ARI coming to public clinics, there were 175 (80%) given antibiotics [96]. In a study conducted in Tehran, Iran in 2006 in three different settings of ambulatory care (private offices, general health facilities, and hospital-based clinics), an antibiotic prescription rate of more than 80% in children with ARI was reported. And there was no difference between the antibiotic prescription rate made by general practitioners (87.8%) and that made by paediatricians (83.9%) (p value > 0.05) [103]. Likewise, in the United States, in the period 1998 -1999, around 80% of children with non-pneumonia ARI received antibiotics [104].

Lower prescription rates have been reported from studies that were specifically aimed at restricting antibiotic use and from countries such as The Netherlands, which have very stringent antibiotic use policies [106, 107]. In a study conducted from 2007 to 2008 in the outpatient clinic of a children's hospital in Tehran, Iran, the antibiotic prescription rate in 0-15 year-old children with ARI was rather low, at 33%, which may be the result of implementing and monitoring a new ARI treatment algorithm in this hospital [106]. And in a study in Holland, which calculated the antibiotic prescription rate among 0–15 year old children with respiratory infections coming to general practitioners, approximately 25% of children with ARI were given antibiotics [107].

The most commonly used antibiotics in our study were the broad spectrum/new generation oral preparations such as amoxicillin-clavulanic acid, cefuroxime, cefixime or cefaclor, which are not recommended in the National Therapeutic Guideline of the Ministry of Health (Appendix H) or in the WHO's IMCI guidelines (Appendix F) [12, 234].

In a 2010 review article published by the Cochrane library, the authors reached the conclusion based on three studies involving 3952 children that amoxicillin had similar failure and cure rates as cotrimoxazole in the treatment of outpatients with non-severe pneumonia, and based on limited data, that amoxicillin-clavulanic acid and cefpodoxime may be the alternative second line options [102]. For GAS pharyngitis, in a systematic review published in Cochrane library in 2011, it was concluded that penicillin is still the first choice antibiotic and there was no significant difference between cephalosporins and penicillin in terms of symptom resolution [241]. Nelson's Textbook of Paediatrics (2011) and the 2012 clinical practice guideline for pharyngitis from the Infectious Diseases Society of America (IDSA) also point out that penicillin or amoxicillin is strongly recommended with high quality of evidence in the treatment of streptococcal pharyngitis because of low cost and high efficacy. In addition, penicillin resistant (primary or secondary) GAS have not been documented so far. Oral cephalosporins are indicated in penicillin-allergic individuals (except those with immediate type hypersensitivity to penicillin) and narrow-spectrum cephalosporins, such as cefadroxil or cephalexin, are preferred over broad-spectrum cephalosporins, such as cefaclor, cefuroxime, cefixime, cefdinir, and cefpodoxime, as the latter have higher minimum inhibitory concentration (MIC) for GAS, and are more expensive and more likely to have side effects [99, 236] than the former.

Our study found no evidence for penicillin usage, and only a very small number of patients received amoxicillin alone (17/563 - 3%) or cefadroxil (7/563-1.2%). Most patients were prescribed broader spectrum antibiotics such as amoxicillin-clavulanic acid (257/563-45.6%), cefuroxime (122/563-22%), cefixime (11.4%), and cefaclor (46/563-8.2%) and the spectrum of antibiotics was almost similar between URI and LRI.

The results from our study are in agreement with a study carried out in 2007 in the outpatients clinics of a university-affiliated children's hospital in Tehran, Iran, which also indicated that amoxicillin-clavulanic acid was the most frequently used antibiotic [106], and with the study in Ba Vi, Vietnam in 2007 which showed that the most commonly used antibiotics were amoxicillin or penicillin (58%), and cephalosporins (25%) [96]. In contrast, in a study conducted also in Ba Vi province, Vietnam, but in the year of 1999, ampicillin was prescribed the most (74%), followed by penicillin (12%), amoxicillin (11%), erythromycin (5%) and tetracycline (4%) and no use of cephalosporins was recorded [95]. This may be explained by the different time points at which the studies were conducted and the different levels of health care involved (referral hospital vs. small town health stations), which could have influenced the choice of antibiotics. Also, in the 1990s, fewer broad spectrum or newer generation oral antibiotics (such as amoxicillin-clavulanic acid, second and third generation cephalosporins) were available in Vietnam, as they were too expensive, suggesting that the overuse of these broad spectrum preparations is a relatively new phenomenon.

In summary, antibiotic prescriptions for patients with ARI in CH1 were often not in accordance with treatment guidelines, in terms of both the indications for and the spectrum of antibiotics although CH1 has implemented a number of measures to

control the inappropriate use of these drugs. There is a "drug and therapeutic committee" in CH1, which is in charge of guideline development, drug utilization reviews and training. Since the 1990s, CH1 has promulgated its own ARI therapeutic guidelines (Appendix G) which are based on the National Therapeutic Guideline of the Ministry of Health and the WHO's IMCI guidelines and are revised every two years or when there are any updated guidelines issued. Trainings on such hospital treatment guidelines have been provided annually for all physicians in the hospital. Monitoring and evaluations of rational drug use have been deployed monthly. However, the rate of doctor adherence to ARI therapeutic guidelines is extremely low. This may be explained by several factors: (1) high number of outpatients in CH1 renders doctors with very little time (around 3 minutes for every patient) for history and examination to make diagnostic and therapeutic judgements; (2) there are no available rapid antigen detection tests (like rapid test for GAS in pharyngitis, or rapid tests for pneumococci, influenza viruses) in CH1 to aid physicians in differentiating bacterial from viral infections; (3) patients' and parents' expectations when visiting a referral hospital outpatient clinic are an important factor affecting the doctor's decision on antibiotic prescribing; (4) incentives from pharmaceutical companies given to doctors who prescribe antibiotics, particularly the new generation ones, contribute to a very high rate of use of these new and expensive antibiotics in outpatient department of CH1. The over-prescription of antibiotics can contribute to a significant increase in bacterial resistance, which is now already detected at alarming rates in Vietnam and the region. It also accounts for a great upsurge in expenses within the healthcare systems.

In our study, 58/563 (10.3%) patients received corticosteroids, of whom only 10/58 (17.3%) were diagnosed with asthma. The other (48/58 - 82.7%) patients were given a non-asthma diagnosis such as pharyngitis, rhino-pharyngitis, bronchitis, or bronchiolitis, all of which would not benefit from corticosteroids. 13/122 (10.7%) patients with bronchiolitis were prescribed with corticosteroids, which have been shown to be clinically ineffective for this indication in a Cochrane's systematic review in 2010 [242]. According to Nelson's Textbook of Paediatrics (2011), corticosteroids are not recommended for children with bronchiolitis, particularly for previously healthy infants with human RSV [99].

In addition, 99/122 (81.1%) and 195/262 (74.4%) patients with bronchiolitis and bronchitis, respectively, were given oral bronchodilators such as salbutamol or terbutaline, which have shown no benefit in terms of reducing the need for admission, shortening illness duration at home (in bronchiolitis), or relieving cough (in bronchitis). These medications have been reported to bring about severe side effects such as tachycardia, shakiness, nervousness and tremors [243, 244]. In addition, WHO strongly recommends that oral salbutamol (the most common bronchodilator in our study) should not be used for treatment of acute wheeze and bronchoconstriction in children except where inhaled salbutamol is not available [238].

Seventy percent (394/563) of patients received cough syrups. This is somewhat similar to the study in Iraq, which demonstrated that around 61% of children with ARIs were prescribed with cough syrups [232]. Among patients receiving cough syrups in our study, 80.6% (317/394) used herbal remedies such as syrup Pectol or Zecuf. The remaining 19.4% (77/394) were prescribed with over-the-counter (OTC) cough products such as syrup Atussin and Iyafin, which contain chlorpheniramine

maleate, dextromethorphan hydrobromide, guaifenesin (Atussin and Iyafin), and pseudoephedrine (Iyafin).

With regard to herbal cough products, according to WHO's IMCI guidelines, children with cough could be treated with a safe remedy, such as warm and sweet drinks or herbal remedies to relieve throat pain and cough [12]. Simple gargling with warm salt water is reported by textbooks to provide local relief in children with acute pharyngitis [99]. A review article published in the Cochrane Library in 2012 could not advocate or reject the use in sore throat of Chinese traditional herbs, which have similar compositions as herbal cough syrups used in our study, as the reviewed studies did not employ methodologies which were rigorous enough to support either conclusion [245]. The same findings were found by L Jiang, who could not demonstrate that the Chinese medicinal herbs were efficacious in acute bronchitis [246, 247].

Regarding OTC cough products, in a Cochrane systematic review from 2012, which included 6 randomised controlled trials in children, not enough evidence was found to support the effectiveness of OTC medicines in acute cough in children [248]. WHO's IMCI guidelines do not recommend the use of commercial cough and cold products (like Atussin and Iyafin), as they can suppress cough which is a physiological mechanism to eliminate respiratory secretions [12].

In Nelson's Textbook of Paediatrics 2011, the authors pointed out that OTC cough and cold products should not be used for infants and children under 2 years of age because of the lack of direct evidence for effectiveness and the potential for unexpected adverse effects of these medications [99].

In brief, although there is not enough evidence to support the efficacy of cough syrup with herbal composition in ARI in children, this medication is currently still the drug of choice to relieve throat pain and cough in CH1 in compliance with the WHO's guideline. For OTC cough products, in spite of no recommendation for their use in children, there was still a number of around 20% of cases being prescribed with these products in our study.

Around one-tenth (66/563) of patients in our study were given antihistamines, all of which were the first-generation antihistamines such as chlorpheniramine, dexchlorpheniramine, or alimemazine. 80.6% (454/563) of patients in our study had symptom of runny nose, and according to M. Kliegman et al, antihistamines can reduce rhinorrhoea by 25 – 30% in patients with common cold or rhino-pharyngitis [99]. However, there is no good evidence for or against the effectiveness of antihistamines in acute cough [248]. Moreover, this medication has a sedative effect on children and can make bronchial secretions become dry and is not indicated in LRI (bronchitis, bronchiolitis) [99] which accounted for 28.8% (19/66) of patients using antihistamines in our study. In addition, 53% (35/66) of children under 2 years old in our study received antihistamines, which have never been adequately studied in paediatric age groups, particularly in children below 2 years old [227]. Therefore, it can be seen clearly that for most of the antihistamine use in outpatients with ARI in CH1, there was insufficient evidence for efficacy as well as safety. In comparison, the prescription rate of antihistamines was even higher, at 64.7%, in children with ARI coming to outpatient departments in public hospitals in Tehran, Iran [103].

Physicians gave mucolytic agents to 11% of children in our study in the belief that these medications could loosen mucus from the respiratory tract. However, these

mucolytic drugs seem to have limited effect in symptomatic treatments for ARI in children and are not yet recommended for children under the age of two as there was very little data available to evaluate safety in infants younger than two years [249]. In our study, 45% (28/62) of patients using mucolytic agents were below 2 years. Hence, expectorant usage in our study was, more often than not, inappropriate and should not have been recommended.

In summary, this chapter provides a description of the presentation and management characteristics of outpatients with ARI in dedicated respiratory examination rooms in CH1. LRI outnumbered URI by almost 3 times and most of the patients had good outcomes after one week. In most cases, the management of ARI was not in accordance with the WHO's IMCI guidelines, the National Therapeutic Guidelines of the Ministry of Health nor with the most recent evidence-based guidelines for ARI and with those of paediatric textbooks (Table 3-5). Antibiotics, in terms of both indication and choice of antibiotics, were prescribed more frequently and with a broader spectrum than suggested for almost all patients. Other drugs were also prescribed more often, and for other indications and age groups than recommended in guidelines. This appropriateness of antibiotic use will be discussed in more detail in the following chapters.

Table 3-5 Summary of the overuse of medications in ARI children in our study

Medications used in ARI	Rate of medication use in our study	Recommendations from textbooks/ evidence-based guidelines
Antibiotics	99.6% (561/563) (pneumonia was clinically diagnosed in only 1.2% of patients)	Antibiotics are not indicated in most ARI in children, apart from pneumonia and bacterial pharyngitis [99-102, 235, 236]
Types of antibiotics prescribed	Amoxicillin-clavulanic acid (45.6%), cefuroxime (22%), cefixime (11.4%), and cefaclor (8.2%)	Penicillin or amoxicillin is the antibiotic of choice in pneumonia and bacterial pharyngitis [99, 102, 236, 241]
Herbal cough syrups	56.3% (317/563)	Current evidence could not advocate or reject the use in sore throat of herbal cough syrups [245]
OTC cough syrups	13.7% (77/563)	OTC cough syrups are not recommended for ARI [12].
Oral corticosteroids	10.3% (58/563), of whom 82.7% (48/58) were non-asthmatic diagnoses such as pharyngitis, rhino-pharyngitis, bronchitis, or bronchiolitis	Oral corticosteroids are not recommended for children with pharyngitis, rhino-pharyngitis, bronchitis, or bronchiolitis [99, 242]
Bronchodilators	57.6% (324/563), of whom 4% (13/324) had asthma, 92% (297/324) had LRI including bronchiolitis or bronchitis or pneumonia	Oral salbutamol should not be used for treatment of acute wheeze and bronchoconstriction in children with LRI including bronchiolitis or bronchitis or pneumonia [238].
Antihistamines	11% (66/563)	There was insufficient evidence for efficacy and safety of antihistamines in ARI, particularly in children under 2 [99, 227, 248].
Mucolytic agents	11% (62/563), of whom 45% (28/62) were below 2 years	Mucolytic drugs are not yet recommended for children under the age of two [249]

Chapter 4

Identification of respiratory pathogens in outpatients with acute respiratory infections and in healthy children in Children's Hospital 1

4.1 Introduction

Results from Chapter 3 and various studies from all parts of the world [95, 96, 103, 104, 232] indicate that over-prescription of antibiotics for children with mild acute respiratory infections (ARI) in an outpatient setting is common. One of the main reasons for this is that it is difficult for physicians to rule out bacterial pathogens in mild ARIs, which are mostly caused by viruses [1-4, 7, 16, 36, 52]. They therefore tend to give antibiotics to treat all possible treatable diagnoses including a bacterial aetiology.

Determining the presence of causative pathogens of ARI could play an important role in guiding treatment of these infections, particularly with respect to the appropriate use of antibiotics. Although viruses are recognised as the predominant aetiologic agents in many studies [1-4, 7, 16, 36, 52], very little is known about the aetiology (especially bacterial agents) in children with ARI in outpatient settings in Vietnam. In this chapter, we aim to describe the presence of respiratory pathogens as well as the asymptomatic carriage of these organisms in children with or without ARI attending the outpatient department in Children's Hospital 1 (CH1), Ho Chi Minh City, Vietnam.

4.2 Materials and methods

Data for this chapter are derived from two prospective descriptive studies conducted in the outpatient department of CH1: one study carried out in patients with ARIs in

respiratory examination rooms from the beginning of February 2009 to the end of January 2010 (03AV), and another study in healthy children who came to the vaccination room from the beginning of December 2010 to the end of November 2011 (01RS). Details of the two studies' designs, materials and methods are described in chapter 2.

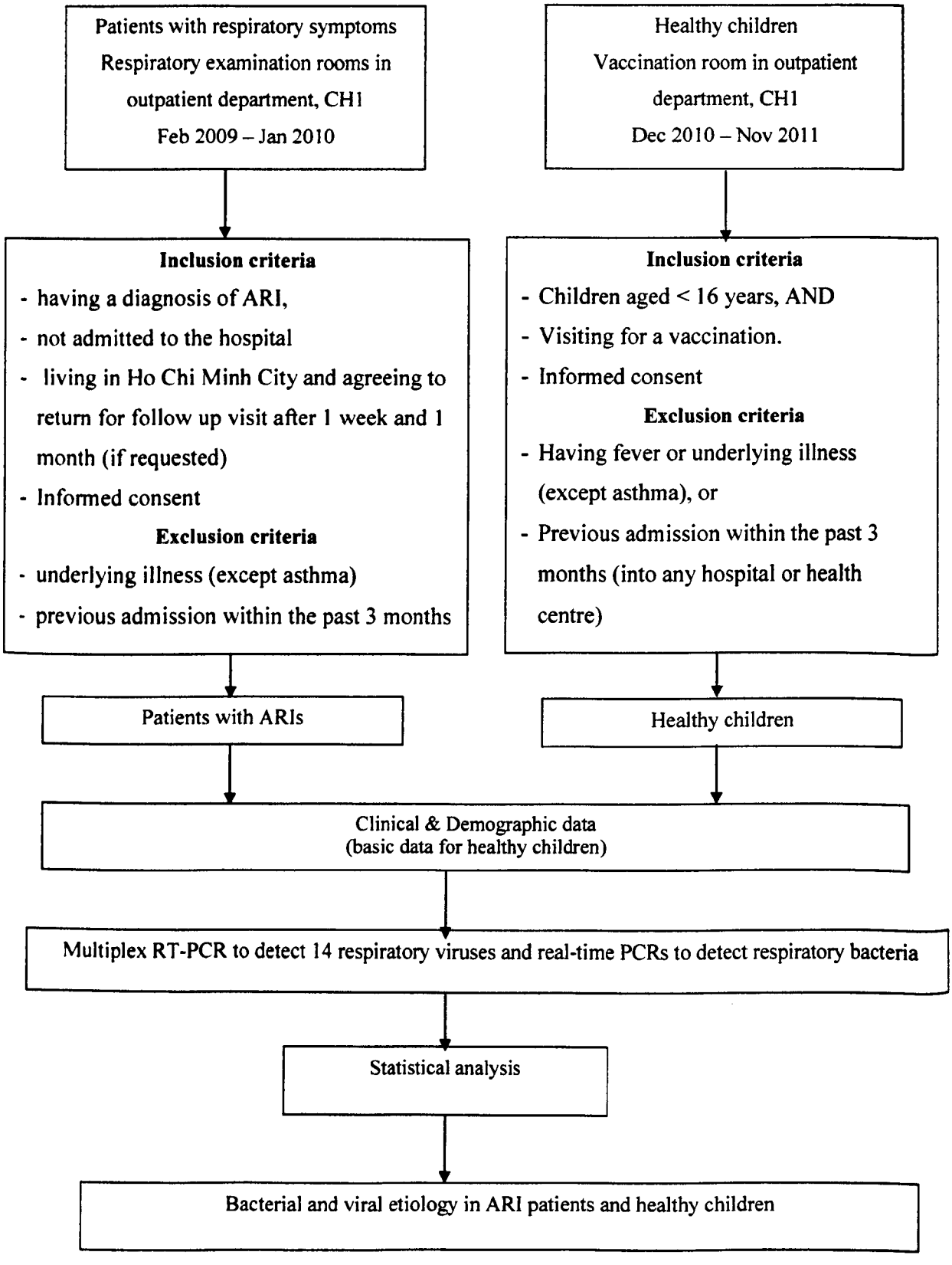

Figure 4-1 Study flow diagram

4.3 Results

4.3.1 Basic demographics of ARI outpatients and healthy children

During a period of one year for each study, there were 563 patients with ARI and 255 healthy children enrolled. Gender and age distributions among ARI outpatients and healthy children are shown in Table 4-1. There was a relative over-representation of children between 2 and 5 years old in the patient group, and only a few ARI patients were older than 5 years. The proportion of males was similar between the two study populations (56.1%).

In the group of healthy children, 39 (15.3%) were reported to have mild respiratory symptoms (as this was not an exclusion criterion), which were not deemed to require a doctor's visit or treatment or to be an exclusion criterion for vaccination.

Table 4-1 Basic demographics of ARI and healthy children

Characteristics	ARI patient N = 563	Healthy children n = 255	P value
Median age in years (IQR)	1.96 (1.05-3.18)	1.78 (.94 – 4.72)	0.5 [1]
≤ 1 year, n (%)	137 (24.3)	69 (27.1)	0.46 [2]
1-≤ 2 years, n (%)	148 (26.3)	68 (26.7)	0.98 [2]
2- ≤ 5 years, n (%)	251 (44.6)	56 (22.0)	<0.001 [2]
>5 years, n (%)	27 (4.8)	62 (24.3)	<0.001 [2]
Male, n (%)	316 (56.1)	143 (56.1)	1.0 [2]

[1] Mann-Whiney test; [2] Chi-square test

In order to identify aetiologic agents in ARI patients, we collected NPAs and a combined nasal and throat swab (two separate swabs, combined in one VTM vial: NTS). From healthy children, only NTS samples were collected as these are easier to take, less invasive and therefore cause less discomfort. This was also done based on

results of a study by Do et al, which showed that PCR results from NTS were similar to those from NPA [16]. This will be discussed in more detail below. For the remainder of this chapter, we will use results from the NTS when presenting data comparing detection rates between ARI patients and healthy controls.

4.3.2 *Streptococcus pneumoniae* and *Haemophilus influenzae* in ARI patients and healthy children

Table 4-2 indicates that detection rates of SP were unexpectedly high, but very similar between ARI patients (98.4%) and healthy children (96.9%). For Hin, the prevalence was higher in healthy subjects (25.1%) than in those with ARI (12.3%) (Chi-square test, p value = 0.000). Both were identified with significantly higher loads in ARI patients compared to healthy children.

The detection rates of these two bacteria were not significantly different between symptomatic and asymptomatic healthy children, but the bacterial load (as expressed by the Cp value, which is defined as the number of cycles required for the fluorescent signal to cross the threshold and is inversely proportional to the amount of target nucleic acid of pathogens in the samples) of SP was significantly higher in the group of symptomatic subjects (Mann-Whitney test, p value = 0.005).

The Cp values of these two bacteria in both ARI patients and healthy children had very similar distributions, although the peak of Cp values in the patient group was lower than that in the group of healthy children (Figure 4-2 and Figure 4-3).

Among ARI patients, there were no differences regarding detection rate and bacterial loads of Hin between LRI and URI. Similarly, SP was detected at the same rate in both LRI (98.3%) and URI (98.1%). The bacterial loads of SP in patients with LRI

were significantly higher than those in patients with URI (Mann-Whitney test, p value = 0.02) (Table 4-4).

When stratified into age groups, in both ARI patients as well as in healthy children, the detection rate of Hin was higher among children older than five years of age (Chi-square test, p value < 0.05) (Table 4-5). The load was not significantly different between the two age groups. This may explain the observed differences in detection rates between patients and healthy children, because the healthy children included a greater proportion of children aged > 5.

In contrast, the load of SP was significantly higher in children less than 5 years of age in both sick and healthy children (Kruskal-Wallis test, p value < 0.05) while difference in detection rates between the two age groups was not statistically significant at the 0.05 level. The prevalence and bacterial loads of both SP and Hin were not different when these bacteria existed alone or co-existed with other pathogens (Table 4-6).

Among ARI patients with SP at Cp-value lower than the 50^{th} percentile, and particularly lower than the 10^{th} percentile, there were significantly higher proportions of children with LRI than among those children with Cp-values greater than 50^{th} percentiles (78% versus 67%, Kruskal-Wallis Test, p value=0.03 (Table 4-3). However, Cp-values of less than the 25^{th} percentile were also found at significantly higher rate in patients younger than 5, and children between 1 - 2 years of age, than in children older than 5. When we performed logistic regression to examine the association of a set of variables including age, sex, Cp-values of SP, and days of illness with the presence of LRI, there was no association between low Cp- values of SP and LRI whereas age and days of illness remained significantly associated with the

presence of an LRI (Table 4-7). With respect to the detection rate of other pathogens, there were no significant differences between patients identified with SP at low Cp-values (< 10^{th} percentile) and those with SP at higher Cp-value (25^{th} - 50^{th} percentile and > 50^{th} percentile).

For Hin, there were no differences in terms of severity, age, and the presence of other pathogens between different percentiles of Cp-values. There were 4 outliers in whom Hin was detected at very low Cp-values (from 20.7 to 27.4). These 4 patients had no special characteristics of note. They were all younger than 5 years old and were clinically diagnosed with LRI. All four patients were found to be infected with either single virus (PIV-4 or hRV) or double viruses (PIV-2 and hCoV or EV and MPV) in addition to Hin.

All these results were obtained from NTS samples. When we repeated the analysis for NPA specimens, there was no difference in the severity of ARI, age, or presence of other pathogens between patients infected with SP or Hin at high loads and those at very low loads. There were a substantial number (124) of patients who were positive with SP in NTS but negative with SP in NPA. When we compare SP negative in NPA but positive in NTS with SP positive in both sampling methods, no significant differences were found between the two groups in terms of the rates of LRI and the presence of other pathogens. However, it appears that patients aged over 2 years had significantly lower detection rates for SP in NPA and patients negative for SP in NPA had significantly lower loads of SP in NTS (Table 4-8).

We conclude that both SP and Hin were detected at a high frequency in the respiratory tract of children with and without symptoms of ARI. We also find an association of younger ages, and LRI with lower Cp-values for SP in ARI patients; of

subclinical respiratory symptoms with lower Cp-values for SP in healthy children, but no association between the presence of other pathogens and the Cp-values. These associations may indicate increased respiratory secretions and shedding during infection or inflammation of the respiratory mucosa, but because the sampling site is the upper respiratory tract which is known to be colonised by SP, while SP is a causative agent of pneumonia in the lower respiratory tract we cannot draw any conclusions about the contribution of SP to the clinical syndrome of the patients.

In the rest of this chapter we have therefore excluded Hin and SP as pathogens when discussing the various detection rates.

Table 4-2 *Streptococcus pneumoniae* and *Haemophilus influenzae* in ARI patients and healthy children

Organism detected	ARI patients N=563	Healthy children n= 255			P1 value	P2 value
		Asymptomatic n1= 216	Mildly respiratory symptomatic n2=39	Total		
Hin, n(%)	69 (12.3)	52 (24.1)	12 (30.8)	64 (25.1)	0.38[(1)]	0.000 [(1)]
Cp-value Hin, median (IQR)	35.7 (33.1-37.3)	37.9 (35.6-40)	38.3 (35.5-40)	37.9 (35.6-40)	0.8[(3)]	0.000[(3)]
SP, n(%)	553 (98.4)	209 (96.8)	38 (97.4)	247 (96.9)	1.0[(2)]	0.2 [(1)]
Cp-value SP, median (IQR)	24.9 (23.1-27.1)	30.2 (27.7-33.3)	28.8 (25.3-30.8)	29.9 (27.5-32.9)	0.005[(3)]	0.000[(3)]

[(1)]:Chi-square test; [(2)]: Fisher's Exact test; [(3)]: Mann-Whitney test

P1-value was calculated between asymptomatic children and mildly respiratory symptomatic children
P2-value was calculated between ARI children and total healthy children
Cp-value: crossing point –value
SP: *Streptococcus pneumoniae*; Hin: *Haemophilus influenzae*

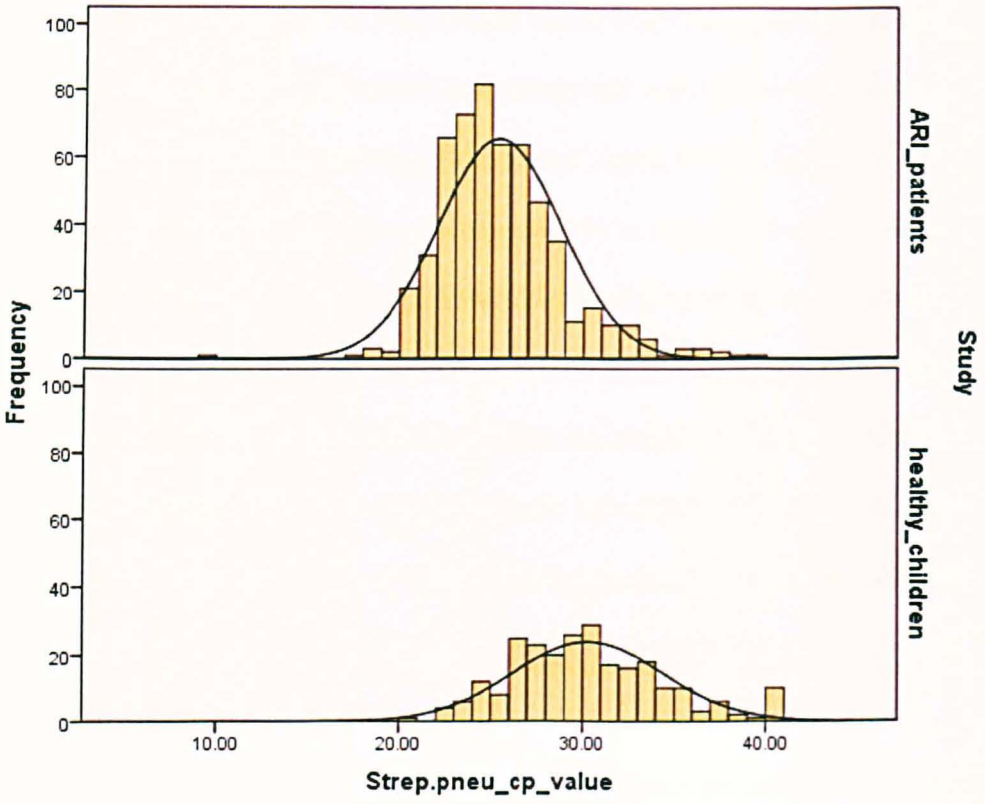

Figure 4-2 Distribution of Cp- values of SP in ARI patients and healthy children.

Line represents the normal distribution curve with mean and SD of Cp-values in samples

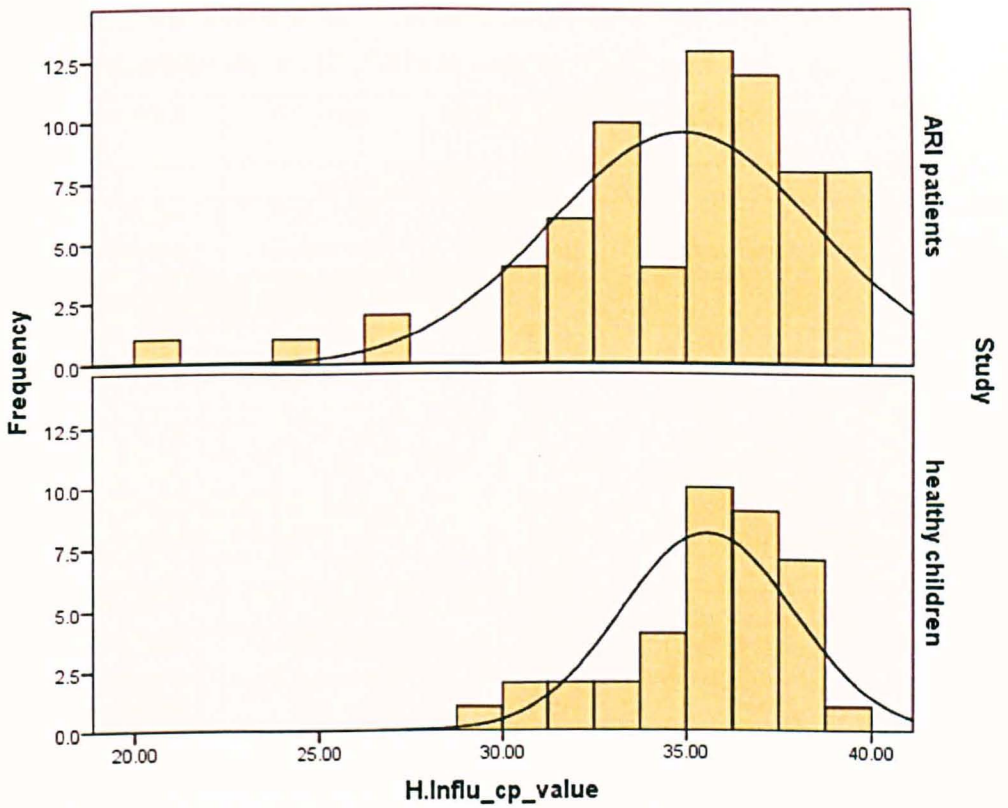

Figure 4-3 Distribution of Cp- value of Hin in ARI patients and healthy children

Line represents the normal distribution curve with mean and SD of Cp-values in samples

Table 4-3 Correlation of age groups, severity of diseases and the presence of other pathogens with different groups of SP, Hin loads (different percentiles of Cp-values of SP, Hin) in ARI patients

	SP, Cp-value				
	< 10th percentile N=56	**10th -25th percentile N= 79**	**25th -50th percentile N = 141**	**>50th percentile N=277**	**P value (among different groups)***
LRI, n (%)	44 (78.6)	61 (77.2)	111 (78.7)	186 (67.1)	0.03
Other pathogens positive, n (%)	41 (73.2)	64 (81)	110 (78)	202 (72.9)	0.4
Age < 1, n (%)	8 (14.3)	17 (21.5)	33 (23.4)	75 (27.1)	0.2
Age 1-2, n (%)	20 (35.7)	33 (41.8)	33 (23.4)	61 (22)	.001
Age 2-5, n (%)	27 (48.2)	29 (36.7)	70 (49.6)	121 (43.7)	0.3
Age > 5, n (%)	1 (1.8)	0 (0)	5 (3.5)	20 (7.2)	0.03
Age < 5, n (%)	55 (98.2)	79 (100)	136 (96.5)	257 (92.8)	0.03
	Hin, Cp-value				
	< 10% percentile N=7	**10% -25% percentile N= 10**	**25%-50% percentile N = 18**	**>50% percentile N=34**	**P value (among different groups)***
LRI, n (%)	5 (71.4)	5 (50)	10 (55.6)	29 (82.9)	0.09
Other pathogens positive, n (%)	4 (57.1)	8 (80)	15 (83.3)	22 (65.7)	0.4
Age < 1, n (%)	0 (0)	0 (0)	0 (0)	6 (17.6)	0.08
Age 1-2, n (%)	2 (28.6)	0 (0)	1 (5.6)	8 (23.5)	0.1
Age 2-5, n (%)	4 (57.1)	7 (70)	15 (83.3)	18 (52.9)	0.2
Age > 5, n (%)	1 (14.3)	3 (30)	2 (11.1)	2 (5.9)	0.2
Age < 5, n (%)	5 (83.3)	8 (72.7)	16 (88.9)	32 (94.1)	0.3

* Kruskal-Wallis Test

Table 4-4 Prevalence and bacterial load of SP and Hin between LRI and URI

Pathogens	URI	LRI	P value
	n= 154	n= 409	
Hin, n(%)	21 (13.6)	48 (11.7)	0.6 (1)
Cp-value Hin, median (IQR)	34 (33-37)	36 (33-38)	0.2 (2)
SP, n(%)	151 (98.1)	402 (98.3)	1.0 (1)
Cp-value SP, median (IQR)	25.5 (23.5-27.5)	24.6 (23.1-26.8)	0.02 (2)

(1): Chi-square test; (2): Mann-Whitney test;

Cp-value: crossing point–value;

SP: *Streptococcus pneumoniae*; Hin: *Haemophilus influenzae*

Table 4-5 Prevalence and bacterial load of SP and Hin by age groups

Pathogen identified	≤ 5 years	>5 years	Total	P value (among different age groups)
ARI patients	**N=536**	**N=27**	**N=563**	
Hin, n(%)	61 (11.4)	8 (29.6)	69 (12.3)	0.01 [1]
Cp-value Hin (IQR)	35.9(33.2-37.5)	33.9 (31.5-36.7)	35.7 (33.1-37.3)	0.2 [2]
SP, n(%)	527 (98.5)	26 (96.3)	553 (98.4)	0.4 [1]
Cp-value SP, (IQR)	24.8 (23.1-26.9)	27.4 (25.3-30.7)	24.9 (23.1-27.1)	0.000 [2]
Healthy children	**N=193**	**N=62**	**N=255**	
Hin, n(%)	40 (20.7)	24 (38.7)	64 (25.1)	0.008 [1]
Cp-value Hin (IQR)	37.9 (35.9-40)	37.7(35.4-40)	37.9 (35.6-40)	0.7 [2]
SP, n(%)	185 (95.9)	62 (100)	247 (96.9)	0.2 [1]
Cp-value SP (IQR)	29.5 (27.1-32.7)	30.8 (28.4-34)	29.9 (27.5-32.9)	0.01 [2]

[1]: Chi-square test; [2]: Kruskal-Wallis Test

Cp-value: crossing point - value

SP: *Streptococcus pneumoniae*; Hin: *Haemophilus influenzae*

Table 4-6 Prevalence and bacterial loads of SP and Hin in relation to the presence of other viruses and atypical bacteria that were tested for in the study

	Other pathogens		P value
	Positive n= 426	**Negative n=137**	
Hin, n (%)	49 (11.5)	20 (14.6)	0.4[1]
Cp-value Hin, median (IQR)	35.3 (33.1-37.1)	36.5 (33.5-38.4)	0.2 [2]
SP, n (%)	417 (97.9)	136 (99.3)	0.7 [1]
Cp-value SP, median (IQR)	24.8 (23.1-26.7)	25.3 (23.4-27.8)	0.09 [2]

[1]: Chi-square test; [2]: Mann-Whitney test;

Cp-value: crossing point – value;

SP: *Streptococcus pneumoniae*; Hin: *Haemophilus influenzae*

Table 4-7 Logistic regression of factors associated LRI

	B	S.E.	Wald	df	P value	OR	95% C.I for OR	
Age in years	-.305	0.064	23.139	1	0.000	0.737	0.651	0.834
Sex	-.114	0.029	0.328	1	0.567	0.892	0.603	1.320
SP, Cp-values	-.038	0.200	1.657	1	0.198	0.963	0.909	1.020
Days of illness < 3	-.499	0.201	6.125	1	0.013	0.607	0.409	0.901
Constant	2.952	0.766	14.824	1	0.000	19.153		

Table 4-8 Comparison of patients with SP negative NPA and SP positive NPA regarding patient age, rate of LTI, SP loads and rates of co-detection of other pathogens

	SP negative in NPA and positive in NTS N=124	**SP positive in both NPA and NTS N= 309**	**P value ***
LRI, n (%)	94 (75.8)	224 (72.5)	0.6
Other pathogens positive, n (%)	93 (75)	243 (75.7)	0.9
Age < 1, n (%)	21 (16.9)	77 (24.9)	0.1
Age 1-2, n (%)	25 (20.2)	91 (29.4)	0.06
Age <2, n (%)	46 (37.1)	168 (54.4)	0.002
Age 2-5, n (%)	70 (56.5)	135 (43.7)	0.02
Age > 5, n (%)	8 (6.5)	6 (1.9)	0.03
SP NTS Cp-value <10th percentile	10 (8.1)	29 (9.4)	0.7
SP NTS Cp-value 10th-25th percentile	9 (7.3)	45 (14.6)	0.04
SP NTS Cp-value 25th-50th percentile	23 (18.5)	92 (29.8)	0.02
SP NTS Cp-value >50th percentile	82 (66.1)	143 (46.3)	0.000

*Chi-square test

Cp-value: crossing point – value; SP: *Streptococcus pneumoniae*

4.3.3 Comparison of combined nose-throat swabs with nasopharyngeal aspirates for identification of respiratory causative agents

Table 4-9 shows that the total detection rates of at least one pathogen in NPA specimens and NTS samples were almost identical (75.5% and 75,6%, respectively, McNemar's test, p value = 1.0). Similarly, for total single viral detections (48.8% vs. 52.2%), total single bacterial detections (2.1% vs. 2.8%), as well as co-detections (24.5% vs. 20.6%), there was no significant difference regarding detection rates between the two sampling methods (McNemar test, $p < 0.05$).

For separate pathogens, there were significantly higher detection rates in NPA than in NTS for Flu A, hRV, and BoV (McNemar test, p value < 0.05). In contrast, PIV-3, MP, Hin, and SP were detected more often in NTS than in NPA (McNemar's test, p value < 0.05). For the other pathogens, no significant difference in detection rates was observed between these two respiratory samples. When calculating Kappa scores to measure the level of agreement between the two sampling methods in detecting individual pathogens, we found that in two thirds of pathogens, Kappa scores were equal to or higher than 0.5 indicating moderate to very good agreement between NTS and NPA in identifying separate causative agents (Table 4-9). In addition, when we calculated Kappa score for the whole analysis (Table 4-10), the overall Kappa score was 0.7 which pointed out a substantial agreement between the two methods in identifying any pathogen in our study. Regarding pathogen loads, expressed by Cp values, with the exception of BoV and SP which had significantly higher loads in NTS than in NPA, most other pathogens including Flu A, AdV, hRV, RSV A/B, MPV, PIV-1, PIV-2, PIV-3, PIV-4, CoV, MP, were detected with higher loads in NPA compared to those in NTS (Wilcoxon Signed Rank test, p value < 0.05).

When we combined the results of NPA and those of NTS, Table 4-9 shows that the detection rate of one or more pathogens was almost 87% which was much higher than the rate in NTS or NPA alone.

Table 4-9 Detection rates and pathogen loads of pathogens detected from NTS and NPA

Pathogens	NPA	NTS	Both NPA and NTS	P-value (NPA vs. NTS)	Level of agreement Kappa value
	n= 563	n= 563	n= 563		
Flu A, n(%)	51 (9.1)	39 (6.9)	52 (9.2)	0.02 [1]	Kappa = 0.8
Cp-value Flu A median (IQR)	32.9 (29.7-34.8)	34.4 (33.3-36.0)		0.000 [2]	
Flu B, n(%)	9 (1.6)	5 (0.9)	12 (2.1)	0.34 [1]	Kappa = 0.3
Cp-value Flu B median (IQR)	34.3 (32.0-34.7)	35.5 (34.6-38.4)		0.2 [2]	
EV, n(%)	48 (8.5)	59 (10.5)	79 (14.0)	0.16 [1]	Kappa = 0.5
Cp-value EV median (IQR)	32.7 (31.4-35.0)	33.7 (32.5-36.0)		1.0 [2]	
AdV, n(%)	56 (9.9)	52 (9.2)	83 (14.7)	0.69 [1]	Kappa = 0.4
Cp-value AdV median (IQR)	32.6 (28.9-35.0)	34.6 (31.4-36.3)		0.001 [2]	
hRV, n(%)	180 (32.0)	152 (27.0)	213 (37.8)	0.005 [1]	Kappa = 0.6
Cp-value hRV median (IQR)	30.9 (29.4-32.1)	31.5 (30.2-32.1)		0.000 [2]	
RSV A/B, n(%)	47 (8.3)	54 (9.6)	57 (10.1)	0.09 [1]	Kappa = 0.8
Cp-value RSV A/B median (IQR)	28.6 (27.4-30.6)	31.2 (30.2-32.5)		0.000 [2]	
MPV, n(%)	40 (7.1)	41 (7.3)	57 (10.1)	1.0 [1]	Kappa = 0.6
Cp-value MPV median (IQR)	31.6 (28.0-34.8)	32.6 (30.1-34.2)		0.002 [2]	
PIV-1, n(%)	11 (2.0)	10 (1.8)	12 (2.1)	1.0 [1]	Kappa = 0.9
Cp-value PIV-1 median (IQR)	33.2 (30.0-33.7)	35.0 (31.8-37.6)		0.02 [2]	
PIV-2, n(%)	11 (2.0)	11 (2.0)	14 (2.5)	1.0 [1]	Kappa = 0.7
Cp-value PIV-2 median (IQR)	30.4 (27.1-34.0)	33.0 (31.6-34.0)		0.02 [2]	
PIV-3, n(%)	35 (6.2)	46 (8.2)	52 (9.2)	0.04 [1]	Kappa = 0.7
Cp-value PIV-3 median (IQR)	26.3 (24.5-32.6)	29.8 (26.9-34.2)		0.000 [2]	
PIV-4, n(%)	23 (4.1)	19 (3.4)	24 (4.3)	0.2 [1]	Kappa = 0.9
Cp-value PIV-4 median (IQR)	31.5 (29.9-36.5)	33.5 (32.6-37.0)		0.001 [2]	
CoV, n(%)	19 (3.4)	21 (3.7)	24 (4.3)	0.7 [1]	Kappa = 0.8
Cp-value CoV median (IQR)	29.3 (28.2-33.0)	32.0 (30.8-33.6)		0.003 [2]	
PeV, n(%)	6 (1.1)	4 (0.7)	8 (1.4)	0.7 [1]	Kappa = 0.4
Cp-value PeV median (IQR)	30.9 (29.1-31.7)	30.9 (28.7-32.3)		0.7 [2]	

BoV, n(%)	35 (6.2)	19 (3.4)	38 (6.7)	0.001 [1]	Kappa = 0.6
Cp-value BoV median (IQR)	28.5 (20.8-32.1)	25.1 (22.7-30.3)		0.002 [2]	
BPt, n(%)	2 (0.4)	4 (0.7)	5 (0.9)	0.6 [1]	Kappa = 0.3
Cp-value BPt median (IQR)	37.6	38.5 (36.8-40.0)		NA	
BPp, n(%)	8 (1.4)	11 (2.0)	12 (2.1)	0.4 [1]	Kappa = 0.7
Cp-value BPp median (IQR)	32.9 (28.0-37.2)	33.9 (28.9-40.0)		0.5 [2]	
LP, n(%)	5 (0.9)	0 (0)	5 (0.9)	NA	
Cp-value LP median (IQR)	35.2 (30.3-39.3)			NA	
MP, n(%)	13 (2.3)	25 (4.4)	27 (4.8)	0.004 [1]	Kappa = 0.6
Cp-value MP median (IQR)	33.2 (26.5-36.6)	36.2 (33.6-38.9)		0.01 [2]	
CPn, n(%)	3 (0.5)	3 (0.5)	4 (0.7)	1.0 [1]	Kappa = 0.7
Cp-value CPn median (IQR)	31.0 (26.8-38.5)	30.4 (26.6-30.9)		0.7 [2]	
CPs, n(%)	0 (0)	0 (0)	0 (0)	NA	
Cp-value CPs median (IQR)					
Any pathogen positive, n(%)	425 (75.5)	426 (75.6)	489 (86.9%)	1.0 [1]	
Single viral infection, n(%)	275 (48.8)	294 (52.2)	271 (48.1)	0.2 [1]	
Single bacterial infection , n(%)	12 (2.1)	16 (2.8)	11 (2.0)	0.3 [1]	
Co infection , n(%)	138 (24.5)	116 (20.6)	207 (36.8)	0.07 [1]	
No pathogen , n(%)	138 (24.5)	137 (24.3)	74 (13.1)	1.0 [1]	

[1]: McNemar test; [2]: Wilcoxon Signed Rank test

Cp-value: crossing point - value

FluA: Influenza virus A; FluB: Influenza virus B; RSV A/B: Respiratory Syncytial Virus A and B; PIV1-4: Human parainfluenza viruses 1-4; hRV: Human Rhinovirus; EV: Human Enterovirus; CoV: Human Coronavirus; BoV: Human Bocavirus; MPV: Human Metapneumovirus; PeV: Human Parechovirus; AdV: Adenovirus; MP: *Mycoplasma pneumoniae;* CPn: *Chlamydophila pneumoniae;* CPs: *Chlamydophila psitacci;* LP: *Legionella pneumophila;* BPt: *Bordetella pertussis;* BPp: *Bordetella parapertussis;* SP: *Streptococcus pneumoniae;* Hin: *Haemophilus influenzae*

Table 4-10 Kappa score for level of agreement between NTS and NPA in detecting the whole 14 viruses and 8 bacteria

		NTS		
		Positive	Negative	Total
NPA	Positive	714 [a]	203 [b]	917
	Negative	348 [c]	10901 [d]	11249
	Total	1062	11104	12166

Kappa score = 0.7

[a]: both NTS and NPA positive for a pathogen

[b]: NTS negative and NPA positive for a pathogen

[c]: NTS positive and NPA negative for a pathogen

[d]: both NTS and NPA negative for a pathogen

4.3.4 Viral and bacterial pathogens in ARI outpatients

Out of 563 patients with ARI, 426 (75.6%) were infected with at least 1 respiratory pathogen (Table 4-11). Among these 426 positive cases, there were 575 pathogens identified as single or co-infections. Co-infections were detected in 20.6% (116/563) of cases, one virus was found in 52.2% (294/563) and one bacterium in 2.8% (16/563). Among the 14 viruses we tested, hRV was the most commonly detected pathogen (27%-152/563), followed by EV (10.5%- 59/563), RSV A/B (9.6%-54/563), AdV (9.2%-52/563), and Flu A (6.9%-39/563). In contrast, PeV, Flu B, PIV-1, PIV-2 were detected at very low frequencies, at 0.7% (4/563), 0.9% (5/563), 1.8% (10/563) and 2% (11.563), respectively. Among the human parainfluenza viruses, PIV-3 was most frequently identified at 8.2 % (46/563) (Table 4-11). Of 6 bacteria we screened, MP was the most frequently found bacterium (4.4% - 25/563), followed by BPp (2%-

11/563), Bpt (0.7%-4/563), and CPn (0.5%-3/563). No patients were positive by PCR for CPs or LP.

Considering viral infection separately, the detection rates of one or more viruses in ARI patients was 72.5% (408/563). For bacterial detection, at least one bacterium was found in 7.3% among 563 outpatients with respiratory symptoms.

4.3.5 **Seasonality of pathogens detected in ARI patients**

Detection rates of respiratory pathogens varied from month to month during the year (Figure 4-4). hRV and EV, the two most common pathogens in ARI outpatients, were detected throughout the year with peaks in different months. hRV was found at higher rates from February to April while EV was present most frequently from September to December. AdV was also found during the entire year with peaks in January and October. RSV A/B circulated mainly from July to October, and MPV from October to December. Flu A and B were identified in the rainy season from April to October. Of the PIVs, PIV-3 was the predominant virus detected almost throughout the year. PIV-1, PIV-2, CoV, PeV, and BoV were only found sporadically. For bacteria, MP was the most common bacterium detected predominantly from February to October. Other bacteria such as BPp, Bpt, and CPn were detected only sporadically in our study population.

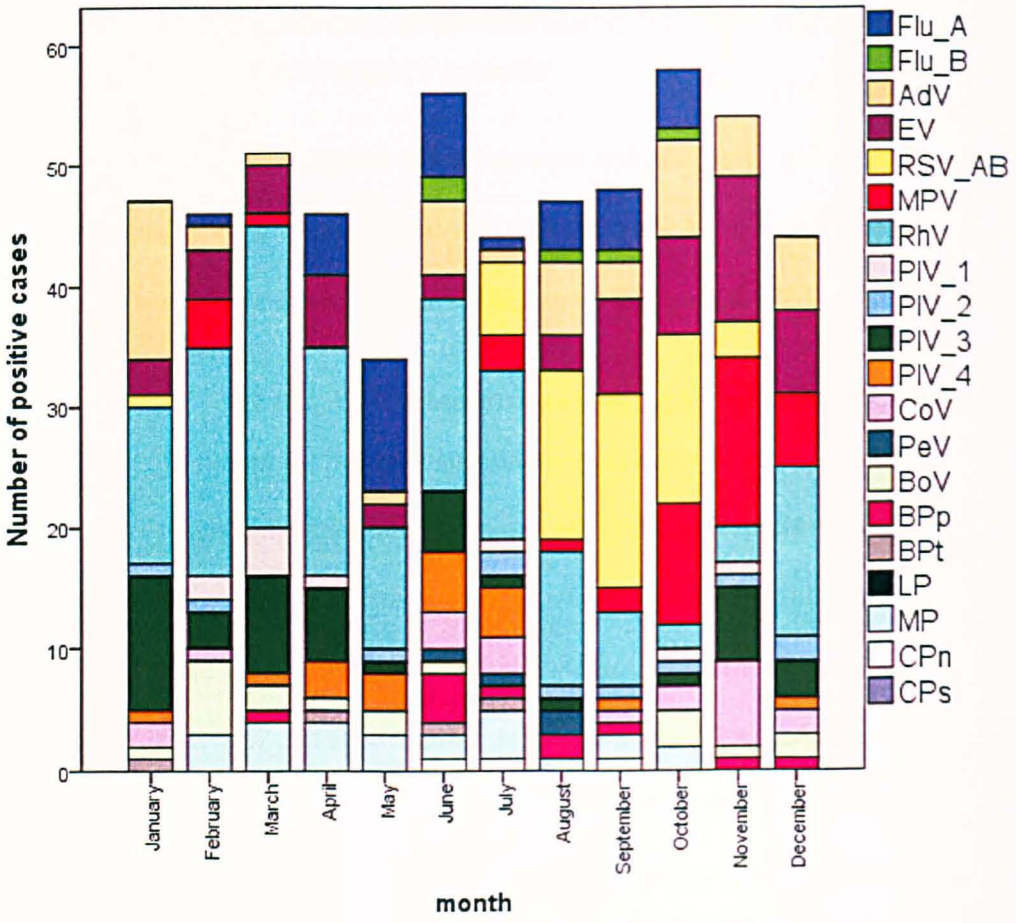

Figure 4-4 Number of respiratory pathogens detected by month

X-axis: time in month; Y-axis: number of positive cases for each pathogen

FluA: Influenza virus A; FluB: Influenza virus B; RSV A/B: Respiratory Syncytial Virus A and B; PIV1-4: Human parainfluenza viruses 1-4; hRV: Human Rhinovirus; EV: Human Enterovirus; CoV: Human Coronavirus; BoV: Human Bocavirus; MPV: Human Metapneumovirus; PeV: Human Parechovirus; AdV: Adenovirus; MP: *Mycoplasma pneumoniae;* CPn: *Chlamydophila pneumoniae;* CPs: *Chlamydophila psitacci;* LP: *Legionella pneumophila;* BPt: *Bordetella pertussis;* BPp: *Bordetella parapertussis.*

4.3.6 Virus and bacteria detections in healthy children

At least 1 respiratory pathogen was detected in 59 (23.1%) out of 255 healthy children. More than 1 pathogen was found in 13/255 children (5.1%) while single viral and single bacterial pathogens were detected in 16.5% (42/255) and 1.6% (4/255) of children, respectively.

As in ARI patients, hRV (12.9%-33/255) and EV (3.9-10/255) were the two most common viruses identified in the respiratory samples in healthy children. The less commonly detected viruses were AdV (2.7%-7/255), CoV (2.4%-6/255), BoV (1.6%-4/255), PIV-3 (1.6%-4/255), MPV (0.4%-1/255), PIV-2 (0.4%-1/255) and PIV-4 (0.4%-1/255). Regarding bacterial pathogens, there were only 6 samples positive for bacteria including MP (3/255- 1.2%), BPt (2/255- 0.8%) and CPn (1/255-0.4%). We did not detect the following pathogens: Flu A, Flu B, RSV A/B, PeV, PIV-1, CPs, BPp, and LP in 255 healthy children visiting the vaccination room.

Among 255 "healthy" children in the study, there were 39 (15.3%) reporting mild respiratory symptoms when asked, of which 20/39 (51%) had cough, 16/39 (41%) had runny nose, 2 had both cough and runny nose, and 1 had nasal congestion. The detection rate of any pathogen in children with mild respiratory symptoms was double that in asymptomatic children (41% versus 20%, Chi-square test, p value <0.001). Although no significant difference was found between the two groups regarding single bacterial infection, the detection rate of single viral and mixed infections in the symptomatic group were markedly higher than in the asymptomatic group (28.2% (11/39) vs. 14.4% (31/216) and 12.8% (5/39) vs. 3.7% (8/216), respectively) (Chi-square test, p value = 0.03). hRV and CoV were significantly more frequently detected in children with respiratory symptoms.

As regards pathogen loads which were deduced from Cp-values, we found that most detected pathogens in children with mild respiratory symptoms had higher viral or bacterial loads than those in asymptomatic children, although all these differences were not significant.

In the asymptomatic group, at least one pathogen was found in 19.9% (43/216), of which co-infection accounted for 3.7% (8/216). As to individual agents, the following pathogens were detected: EV (3.7%-8/216), AdV (3.2%-7/216), hRV (10.6%-23/216), PIV-2 (0.5%-1/216), PIV-3 (1,9%-4/216), PIV-4 (0.5%-1/216), CoV (0.9%-2/216), BoV (0.9%-2/216), BPt (0.9%-2/216), and MP (0.9%-2/216).

4.3.7 **Comparison of pathogens detected in ARI patients and healthy children**

As can be seen in Table 4-11, there was a large difference between pathogen detection in ARI outpatients and that in healthy children. With the exception of single bacterial infections, there were highly significant differences between the two populations in terms of any pathogen positive (75.6% in ARI patients versus 23.1% in healthy children; Chi-square test, p value < 0.001), single viral infections (52.2% vs. 16.5%, Chi-square test, p value < 0.001), as well as co-infections (20.6% vs. 5.1%, Chi-square test, p value < 0.001). With respect to separate pathogens, higher prevalence of the following was found in ARI patients compared to healthy children: Flu A (6.9% vs. 0%, p<0.001), EV (10.5% vs. 3.9%, p=0.002), AdV (9.2% vs. 2.7%, p=0.001), hRV (27% vs. 12.9%, p<0.001), RSV A/B (9.6% vs. 0%, p<0.001), MPV (7.3% vs. 0.4%, p<0.001), PIV-1 (1.8% vs. 0%, p=0.04), PIV-3 (8.2% vs. 1.6%, p<0.001), PIV-4 (3.4% vs. 0.4%, p = 0.007), BPp (2% vs. 0%, p=0.02), and MP (4.4% vs. 1.2%, p=0.02). There were no significant differences between the two study populations regarding the prevalence of PIV-2, CoV and BoV, but these were all

detected at very low frequency. Of note was the fact that Flu A, RSV A/B, and BPp were significantly more prevalent among ARI patients but not found at all in any healthy children.

Regarding pathogen loads, most pathogens identified in ARI patients had higher loads than those in healthy children although statistically significant differences were found only in the commonly detected pathogens hRV, MPV, and PIV-3 (Table 4-11).

Comparison between the pathogens detected in ARI patients and those in healthy children with mild respiratory symptoms showed that detection rates of Flu A, Flu B, EV, AdV, hRV, RSV A/B, MPV, PIV-1, PIV-2, PIV-3, PIV-4, PeV, BPt, BPp, MP were higher in ARI patients than in children with mild symptoms although significant differences were found only in cases of AdV and RSV A/B (p values < 0.05). Likewise, with the exception of EV, BoV, and MP, most pathogens were identified with higher pathogen loads in the ARI group than in the mildly symptomatic group although all these differences were not found statistically significant because of the very low number of positive samples in healthy children. Interestingly, among healthy children with mild respiratory symptoms, higher prevalence of CoV, BoV, and CPn was observed compared to ARI patients. However, these differences did not reach statistical significance.

4.3.8 **Single detection and co-detection in ARI outpatients compared to healthy children**

In ARI patients, the following pathogens were found as co-infection with other agents at significantly higher rates than as single infection: EV, AdV, hRV, MPV, PeV, BoV, BPp, and MP (Fisher's Exact test, p value < 0.05), of which PeV was the only virus which was not detected as a mono-infection but only as a co-infection with other

pathogens. For the rest of the other pathogens, there were no significant differences between detection rates of single infection and mixed infection. Looking at Cp-values, there was no significant difference regarding pathogen loads between single detection and co-detection of all pathogens tested.

Regarding pathogen loads between ARI patients and healthy children, viral loads of hRV in both cases of single and co-detection were significantly higher than those of hRV in healthy children (Mann-Whitney test, p value < 0.05). Likewise, PIV-3 was identified as single infection as well as co-infection with higher pathogen loads in ARI patients than in healthy children (Mann-Whitney test, p value < 0.05). Bacterial load of MP detected alone in ARI patients was similar to that of this bacterium in healthy children. However, when co-existing with other pathogens in ARI patients, MP had lower bacterial load compared to its load in healthy children (Mann-Whitney test, p value $= 0.02$). For other pathogens, no significant difference was observed with respect to pathogen loads between single or co-detection in ARI patients and those in healthy children (Table 4-11).

Table 4-11 Pathogens identified in ARI outpatients and healthy children

	ARI patients				Healthy children				P3 value	P4 value	P5 value
	Single detection n=310	Co detection n=116	P1 value (single detection versus co-detection)	Total N= 563	Asymptomatic n=216	Mildly respiratory symptomatic n=39	P2 value (asymptomatic versus mildly symptomatic)	Total N= 255			
Viruses											
Flu A, n(%)	29 (9.4)	10 (8.6)	1.0[2]	39 (6.9)	0 (0)	0 (0)	NA	0 (0)	0.000 [1]	0.00[1]	0.00[1]
Cp-value Flu A (IQR)	34.4(33.4-36.1)	33.9(32-35.4)		34.4(33.3-36.0)					NA	NA	NA
Flu B, n(%)	2 (.6)	3 (2.6)	0.12 [1]	5 (0.9)	0 (0)	0 (0)	NA	0 (0)	0.33 [1]	0.5[1]	0.03[1]
Cp-value Flu B (IQR)	35.8 (34.7-36.9)	35.5 (34.6-40)		35.5 (34.6-38.4)					NA	NA	NA
EV, n(%)	14 (4.5)	45 (38.8)	0.000[2]	59 (10.5)	8 (3.7)	2 (5.1)	0.65 [1]	10 (3.9)	0.002 [2]	0.9[2]	0.000[2]
Cp-value EV (IQR)	33.9 (31.9-35.6)	33.7(32.6-36.1)		33.7(32.5-36.0)	36 (33.3-37.3)	31.58 (31.5-31.6)	0.2[3]	35.3(31.6-37.0)	0.6 [3]	0.6[3]	0.6[3]
AdV, n(%)	12 (3.9)	40 (34.5)	0.000[2]	52 (9.2)	7 (3.2)	0 (0)	0.60[1]	7 (2.7)	0.001[2]	0.6[2]	0.000[2]
Cp-value AdV (IQR)	34.9 (29.9-35.8)	34.3(31.4-36.8)		34.6(31.4-36.3)	35.7(33.9-36.5)		NA	35.7(33.9–36.5)	0.3[3]	0.3[3]	0.3[3]
hRV, n(%)	97 (31.3)	55 (47.4)	0.003[2]	152 (27.0)	23 (10.6)	10 (25.6)	0.01[2]	33 (12.9)	0.000 [2]	0.00[2]	0.000[2]
Cp-value hRV (IQR)	31.5(30-32)	31.7(30.5-32.5)		31.5(30.2-32.1)	35.9(33.1-36.9)	34.2(32.3-35.9)	0.3[3]	35.6(32.8-36.7)	0.000[3]	0.00[3]	0.001[3]
RSV A/B, n(%)	40 (12.9)	14 (12.1)	0.9[2]	54 (9.6)	0 (0)	0 (0)	NA	0 (0)	0.000[1]	0.00[1]	0.000[1]
Cp-value RSV A/B (IQR)	31.2(29.8-32.4)	31.8(30.6-32.7)		31.2 (30.2-32.5)					NA	NA	NA

MPV, n(%)	23 (7.4)	18 (15.5)	0.02[2]	41 (7.3)	0 (0)	1 (2.6)	0.15[1]	1 (0.4)	0.000 [1]	0.00[1]	0.000[1]
Cp-value MPV (IQR)	32.6(30.1-33.6)	32.7(29.8-35.7)		32.6(30.1-34.2)		38.8		38.8	0.048[3]	0.1[3]	0.1[3]
PIV-1, n(%)	9 (2.9)	1 (.9)	0.3[1]	10 (1.8)	0 (0)	0 (0)	NA	0 (0)	0.04 [1]	0.005[1]	0.3[1]
Cp-value PIV-1, median IQR)	36.5(32.3-37.8)	31.7		35.0(31.8-37.6)					NA	NA	NA
PIV-2, n(%)	9 (2.9)	2 (1.7)	0.7[1]	11 (2.0)	1 (0.5)	0 (0)	1.0[1]	1 (0.4)	0.12 [1]	0.03[1]	0.2[1]
Cp-value PIV-2, median (IQR)	33(31.8-35)	31.8(30.5-33.2)		33.0 (31.6-34.0)	32.5			32.5	0.8[3]	0.6[3]	1.0[3]
PIV-3, n(%)	32 (10.3)	14 (12.1)	0.7[2]	46 (8.2)	4 (1.9)	0 (0)	1.0[1]	4 (1.6)	0.000 [1]	0.00[1]	0.000[1]
Cp-value PIV-3, median (IQR)	29.1(26.8-32.6)	33.2(27.3-35.6)		29.8 (26.9-34.2)	38.9 (37-40)			38.9 (37-40)	0.001[3]	0.003[3]	0.01[3]
PIV-4, n(%)	10 (3.2)	9 (7.8)	0.08[2]	19 (3.4)	1 (0.5)	0 (0)	1.0[1]	1 (0.4)	0.007 [1]	0.02[1]	0.000[1]
Cp-value PIV-4, median (IQR)	33.5(32.2-37.8)	33.5(32.6-36.6)		33.5 (32.6-37.0)	32.6			32.6	0.6[3]	0.5[3]	0.4[3]
CoV, n(%)	11(3.5)	10 (8.6)	0.06[2]	21 (3.7)	2 (0.9)	4 (10.3)	0.006[1]	6 (2.4)	0.3 [2]	0.6[2]	0.02[2]
Cp-value CoV (IQR)	31.6(30.4-33.2)	32.4(31.2-34.0)		32.0 (30.8-33.6)	33.9	35.4 (31.9-39.6)	1.0[3]	33.9 (32.4-38.7)	0.06[3]	0.04[3]	0.2[3]
PeV, n(%)	0 (0)	4 (3.4)	0.005[1]	4 (0.7)	0 (0)	0 (0)	NA	0 (0)	0.3 [1]	NA	0.009[1]
Cp-value PeV (IQR)		30.9(28.7-32.3)		30.9 (28.7-32.3)					NA	NA	NA
BoV, n(%)	6 (1.9)	13 (11.2)	0.000[2]	19 (3.4)	2 (0.9)	2 (5.1)	0.11[1]	4 (1.6)	0.2 [1]	1.0[1]	0.000[1]
Cp-value BoV (IQR)	28.7(22.3-31.1)	24.8(22.3-27.2)		25.1 (22.7-30.3)	33.5	25.3	0.3[3]	29.4 (24.2-34.6)	0.2[3]	0.4[3]	0.2[3]
Bacteria											
BPt, n(%)	1 (.3)	3 (2.6)	0.06[1]	4 (0.7)	2 (0.9)	0 (0)	1.0[1]	2 (0.8)	1.0 [1]	0.6[1]	0.2[1]
Cp-value BPt (IQR)	36.8	40 (37-40)		38.5 (36.8-40.0)	36			36	0.8[3]	1.0[3]	0.6[3]

BPp, n(%)	4 (1.3)	7 (6.0)	0.01[1]	11 (2.0)	0 (0)	0 (0)	NA	0 (0)	0.02 [1]	0.1[1]	0.000[1]
Cp-value BPp (IQR)	30.8 (27.4-36)	37.0(30.6-40)		33.9 (28.9-40.0)					NA	NA	NA
LP, n(%)	0	0	NA	0 (0)	0 (0)	0 (0)	NA	0 (0)	NA	NA	NA
Cp-value LP, median (IQR)											
MP, n(%)	9 (2.9)	16 (13.8)	0.000[2]	25 (4.4)	2 (0.9)	1 (2.6)	0.39[1]	3 (1.2)	0.02 [1]	0.2[1]	0.000[1]
Cp-value MP, median (IQR)	34.9 (31.4-36.6)	37.5(34.6-39.8)		36.2 (33.6-38.9)	32.6	30.99	1.0[3]	31.2 (31-34)	0.051[3]	0.3[3]	0.02[3]
CPn, n(%)	2 (.6)	1 (.9)	1.0[1]	3 (0.5)	0 (0)	1 (2.6)	0.15[1]	1 (0.4)	1.0 [1]	1.0[1]	0.5[1]
Cp-value CPn, median (IQR)	28.7(26.6-30.8)	30.4		30.4		35		35	0.5[3]	0.2[3]	1.0[3]
CPs, n(%)	0	0	NA	0 (0)	0 (0)	0 (0)	NA	0 (0)	NA	NA	NA
Cp-value CPs, median (IQR)											
Any pathogen positive, n(%)				426 (75.6)	43 (19.9)	16 (41)	0.004[2]	59 (23.1)	0.000 [2]		
Single viral infection, n(%)				294 (52.2)	31 (14.4)	11 (28.2)	0.03[2]	42 (16.5)	0.000 [2]		
Single bacterial infection , n(%)				16 (2.8)	4 (1.9)	0(0)	1.0[1]	4 (1.6)	0.4 [1]		
Co infection , n(%)				116 (20.6)	8 (3.7)	5 (12.8)	0.03[2]	13 (5.1)	0.000 [2]		
No pathogen , n(%)				137 (24.3)	173 (80.1)	23 (59)	0.004[2]	196 (76.9)	0.000 [2]		

P3 value: whole ARI patients versus whole healthy children

P4 value: single detection in ARI patients versus whole healthy children

P5 value: Co- detection in ARI patients versus whole healthy children

[1]: Fisher's Exact test; [2]: Chi square test; [3]: Mann-Whitney test

Cp-value: crossing point - value

FluA: Influenza virus A; FluB: Influenza virus B; PIV1-4: Human parainfluenzaviruses 1-4; hRV: Human Rhinovirus; RSV A/B: Respiratory Syncytial Virus A and B; EV: Human Enterovirus; CoV: Human Coronavirus; BoV: Human Bocavirus; MPV: Human Metapneumovirus; PeV: Human Parechovirus; AdV: Adenovirus; MP: *Mycoplasma pneumoniae;* CPn: *Chlamydophila pneumoniae;* CPs: *Chlamydophila psitacci;* LP: *Legionella pneumophila;* BPt: *Bordetella pertussis;* BPp: *Bordetella parapertussis;* SP: *Streptococcus pneumoniae;* Hin: *Haemophilus influenzae*

4.3.9 **Pathogen detections by age group in ARI outpatients and healthy children**

In the ARI outpatients, when stratified by age group (≤ 1 year, 1-≤ 2 years, 2- ≤ 5 years, and >5 years), detection rates of at least one pathogen were significantly different among 4 age groups (Chi-square test, p value < 0.001), of which the lowest rate was found in the over-5-year group, at 40.7% (11/27). Rates of single detection as well as co-detection were not significantly different among four age groups. The proportion of patients with no pathogen found was significantly higher in the over-5-year group compared to other groups (Chi square test, p value < 0.001). For individual aetiologic agents, significant differences among four age groups were observed in case of PeV and BoV, of which PeV was found only in children less than 1 year and BoV was much more prevalent in patients below 2 (Chi-square test, p value < 0.01).

In healthy children, no significant difference among 4 age groups was found in terms of the detection rates of any pathogen, one virus, one bacterium, multiple pathogens as well as separate causative pathogens.

Table 4-12 Identification rates of respiratory pathogens detected in NTS by age group

Pathogens detected	≤ 1 year	1-≤ 2 years	2- ≤ 5 years	>5 years	Total	P value
ARI outpatients	**n =137**	**n= 148**	**n= 251**	**n= 27**	**N=563**	
Flu A, n(%)	5 (3.6)	12 (8.1)	19 (7.6)	3 (11.1)	39 (6.9)	0.3
Flu B, n(%)	0 (0)	1 (.7)	4 (1.6)	0 (0)	5 (.9)	0.4
AdV, n(%)	8 (5.8)	20 (13.5)	23 (9.2)	1 (3.7)	52 (9.2)	0.1
EV, n(%)	10 (7.3)	18 (12.2)	28 (11.2)	3 (11.1)	59 (10.5)	0.6
RSV A/B, n(%)	11 (8.0)	17 (11.5)	26 (10.4)	0 (0)	54 (9.6)	0.3
MPV, n(%)	8 (5.8)	14 (9.5)	19 (7.6)	0 (0)	41 (7.3)	0.3
hRV, n(%)	40 (29.2)	46 (31.1)	59 (23.5)	7 (25.9)	152 (27.0)	0.4
PIV-1, n(%)	4 (2.9)	3 (2.0)	3 (1.2)	0 (0)	10 (1.8)	0.6
PIV-2, n(%)	4 (2.9)	1 (.7)	6 (2.4)	0 (0)	11 (2.0)	0.4
PIV-3, n(%)	18 (13.1)	12 (8.1)	15 (6.0)	1 (3.7)	46 (8.2)	0.08
PIV-4, n(%)	3 (2.2)	7 (4.7)	9 (3.6)	0 (0)	19 (3.4)	0.5
CoV, n(%)	5 (3.6)	6 (4.1)	10 (4.0)	0 (0)	21 (3.7)	0.8
PeV, n(%)	4 (2.9)	0 (0)	0 (0)	0 (0)	4 (.7)	0.006
BoV, n(%)	10 (7.3)	7 (4.7)	2 (.8)	0 (0)	19 (3.4)	0.004
BPp, n(%)	2 (1.5)	4 (2.7)	4 (1.6)	1 (3.7)	11 (2.0)	0.8
BPt, n(%)	1 (.7)	0 (0)	3 (1.2)	0 (0)	4 (.7)	0.6
LP, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
MP, n(%)	7 (5.1)	4 (2.7)	14 (5.6)	0 (0)	25 (4.4)	0.4
CPn, n(%)	1 (.7)	1 (.7)	1 (.4)	0 (0)	3 (.5)	0.9
CPs, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Any pathogen positive, n(%)	110 (80.3)	119 (80.4)	186 (74.1)	11 (40.7)	426 (75.6)	0.000
Single viral infection, n(%)	80 (58.4)	77 (52)	129 (51.4)	8 (29.6)	294 (52.2)	0.053
Single bacterial infection, n(%)	3 (2.2)	4 (2.7)	9 (3.6)	0 (0)	16 (2.8)	0.7
Co infection , n(%)	27 (19.7)	38 (25.7)	48 (19.1)	3 (11.1)	116 (20.6)	0.2
No pathogen , n(%)	27 (19.7)	29 (19.6)	65 (25.9)	16 (59.3)	137 (24.3)	0.000
Healthy children	**n=69**	**n=68**	**n=56**	**n=62**	**N=255**	
Flu A, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Flu B, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
AdV, n(%)	3 (4.3)	3 (4.4)	0 (0)	1 (1.6)	7 (2.7)	0.4
EV, n(%)	2 (2.9)	3 (4.4)	3 (5.4)	2 (3.2)	10 (3.9)	0.9

RSV A/B, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
MPV, n(%)	1 (1.4)	0 (0)	0 (0)	0 (0)	1 (.4)	0.4
hRV, n(%)	9 (13.0)	10 (14.7)	8 (14.3)	6 (9.7)	33 (12.9)	0.8
PIV-1, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
PIV-2, n(%)	0 (0)	1 (1.5)	0 (0)	0 (0)	1 (.4)	0.4
PIV-3, n(%)	2 (2.9)	0 (0)	1 (1.8)	1 (1.6)	4 (1.6)	0.6
PIV-4, n(%)	1 (1.4)	0 (0)	0 (0)	0 (0)	1 (.4)	0.4
CoV, n(%)	3 (4.3)	1 (1.5)	2 (3.6)	0 (00	6 (2.4)	0.4
PeV, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
BoV, n(%)	0 (0)	3 (4.4)	0 (0)	1 (1.6)	4 (1.6)	0.1
BPp, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Bpt, n(%)	0 (0)	1 (1.5)	0 (0)	1 (1.6)	2 (.8)	0.6
LP, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
MP, n(%)	0 (0)	1 (1.5)	1 (1.8)	1 (1.6)	3 (1.2)	0.8
CPn, n(%)	0 (0)	0 (0)	0 (0)	1 (1.6)	1 (.4)	0.4
CPs, n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	NA
Any pathogen positive, n(%)	17 (24.6)	20 (29.4)	12 (21.4)	10 (16.1)	59 (23.1)	0.3
Single viral infection, n(%)	13 (18.8)	15 (22.1)	8 (14.3)	6 (9.7)	42 (16.47)	0.3
Single bacterial infection , n(%)	0 (0)	2 (2.9)	1 (1.8)	1 (1.6)	4 (1.57)	0.6
Co infection , n(%)	4 (5.8)	3 (4.4)	3 (5.4)	3 (4.8)	13 (5.1)	1.0
No pathogen , n(%)	52 (75.4)	48 (70.6)	44 (78.6)	52 (83.9)	196 (76.9)	0.3

FluA: Influenza virus A; FluB: Influenza virus B; PIV1-4: Human parainfluenzaviruses 1-4; hRV: Human Rhinovirus; RSV A/B: Respiratory Syncytial Virus A and B; EV: Human Enterovirus; CoV: Human Coronavirus; BoV: Human Bocavirus; MPV: Human Metapneumovirus; PeV: Human Parechovirus; AdV: Adenovirus; MP: *Mycoplasma pneumoniae;* CPn: *Chlamydophila pneumoniae;* CPs: *Chlamydophila psitacci;* LP: *Legionella pneumophila;* BPt: *Bordetella pertussis;* BPp: *Bordetella parapertussis;* SP: *Streptococcus pneumoniae;* Hin: *Haemophilus influenzae*

4.3.10 **Detection of respiratory pathogens and clinical manifestations of ARIs**

RSV and MPV were both more frequently detected in patients with LRI than in those with URI (p value <0.05). There was no significant difference regarding detection rates of other pathogens between LRI and URI (Table 4-13).

Table 4-13 Detection rates of pathogens detected in NTS in upper respiratory infections (URI) and lower respiratory infections (LRI)

Pathogens detected	URI	LRI	P value
	n= 154	n= 409	
Flu A, n(%)	8 (5.2)	31 (7.6)	0.4 [2]
Flu B, n(%)	3 (1.9)	2 (.5)	0.3
EV, n(%)	17 (11)	42 (10.3)	0.9 [2]
AdV, n(%)	14 (9.1)	38 (9.3)	1.0 [2]
hRV, n(%)	43 (27.9)	109 (26.7)	0.8 [2]
RSV, n(%)	8 (5.2)	46 (11.2)	0.04
MPV, n(%)	4 (2.6)	37 (9.0)	0.02 [1]
PIV-1, n(%)	2 (1.3)	8 (2.0)	0.9 [1]
PIV-2, n(%)	3 (1.9)	8 (2.0)	1.0 [1]
PIV-3, n(%)	11 (7.1)	35 (8.6)	0.7 [2]
PIV-4, n(%)	8 (5.2)	11 (2.7)	0.2 [2]
CoV, n(%)	7 (4.5)	14 (3.4)	0.7 [2]
PeV, n(%)	0 (0)	4 (1.0)	0.5 [1]
BoV, n(%)	2 (1.3)	17 (4.2)	0.2 [1]
BPt, n(%)	2 (1.3)	2 (.5)	0.6 [1]
BPp, n(%)	5 (3.2)	6 (1.5)	0.3 [2]
LP, n(%)	0 (0)	0 (0)	NA
MP, n(%)	4 (2.6)	21 (5.1)	0.3 [1]
CPn, n(%)	1 (.6)	2 (.5)	1.0 [1]
CPs, n(%)	0 (0)	0 (0)	NA

[1]: Fisher's Exact test; [2]: Chi-square test

FluA: Influenza virus A; FluB: Influenza virus B; PIV1-4: Human parainfluenzaviruses 1-4; hRV: Human Rhinovirus; RSV A/B: Respiratory Syncytial Virus A and B; EV: Human Enterovirus; CoV: Human Coronavirus; BoV: Human Bocavirus; MPV: Human Metapneumovirus; PeV: Human Parechovirus; AdV: Adenovirus; MP: *Mycoplasma pneumoniae;* CPn: *Chlamydophila pneumoniae;* CPs: *Chlamydophila psitacci;* LP:

Legionella pneumophila; BPt: *Bordetella pertussis;* BPp: *Bordetella parapertussis;* SP: *Streptococcus pneumoniae;* Hin: *Haemophilus influenza*

Comparing epidemiological and clinical characteristics between patients positive with any pathogen and those without pathogen, Table 4-14 shows that the pathogen-negative-group had a higher median age than the pathogen-positive-group (Mann-Whitney test, p value = 0.004). As for respiratory symptoms, runny nose and nasal congestion were found more prevalent in the pathogen-positive-group (82.9% and 65.5%, respectively) than in the pathogen-negative-group (73.7% and 54.0%, respectively) (Chi-square test, p value < 0.05) while the rate of sore throat in patients without pathogen (18.2%) was significantly higher than that in children infected with at least 1 causative agent (11.0%, Chi-square test, p value = 0.04). There was no significant difference between pathogen-positive-group and pathogen-negative-group in terms of clinical findings except for rhonchi, which was observed more frequently in the pathogen-positive-group (66.9%) compared with the pathogen-negative-group (56.2%) (Chi-square test, p value = 0.03). No significant difference was found between the 2 groups with respect to clinical outcomes after one week.

Among patients positive for at least one pathogen, there was no significant difference between the single-detection group and the co-detection group in terms of epidemiological and clinical characteristics except for the outcome of complete recovery, which was observed more commonly in the single-detection group (27.7%) than in the co-detection group (15.5%) (Chi-square test, p value = 0.01).

Table 4-14 Clinical characteristics by pathogens detected

	Any pathogen positive			No pathogen n=137	P1 value	P2 value
	Single detection N=310	Co-detection n = 116	Total N = 426			
Male, n(%)	177 (57.1)	65 (56)	242 (56.8)	74 (54)	0.9(2)	0.6(2)
Median age in years (IQR)	1.9 (1-3.2)	1.9 (1.1-2.7)	1.9 (1-3.1)	2.4 (1.2-3.6)	0.7(3)	0.004(3)
Clinical characteristics						
Respiratory symptoms						
Cough	308 (99.4)	115 (99.1)	423 (99.3)	136 (99.3)	1.0(1)	1.0(1)
Productive cough	248 (80)	95 (81.9)	343 (80.5)	111 (81)	0.9(2)	0.9(2)
Sore throat	38 (12.3)	9 (7.8)	47 (11)	25 (18.2)	0.3(2)	0.04(2)
Running nose	254 (81.9)	99 (85.3)	353 (82.9)	101 (73.7)	0.5(2)	0.03(2)
Nasal congestion	204 (65.8)	75 (64.7)	279 (65.5)	74 (54)	0.9(2)	0.02(2)
Other symptoms						
Conjunctivitis	7 (2.3)	3 (2.6)	10 (2.3)	6 (4.4)	1.0(1)	0.2(2)
Dyspnoea	37 (11.9)	11 (9.5)	48 (11.3)	16 (11.7)	0.6(2)	1.0(2)
Cyanosis	0 (0)	0 (0)	0 (0)	0 (0)	NA	NA
Diarrhoea	15 (4.8)	3 (2.6)	18 (4.2)	7 (5.1)	0.4(1)	0.8(2)
Rash	6 (1.9)	1 (.9)	7 (1.6)	4 (2.9)	0.7(1)	0.5(1)
Examination on presentation						
Median pulse (IQR)	116 (100-120)	117 (108-120)	116 (106-120)	110 (100-120)	0.4(3)	0.08(3)
Median temperature (IQR)	36.8 (36.3-37)	36.9 (36.4-37.2)	36.8(36.3-37)	36.8 (36.4-37.1)	0.2(3)	0.4(3)
Fever, n (%)	40 (12.9)	17 (14.7)	57 (13.4)	19 (13.9)	0.8(2)	1.0(2)
Median Breath rate (IQR)	35 (30-40)	35 (30-40)	35 (30-40)	35 (30-40)	0.7(3)	0.5(3)
Heart sound normal, n(%)	310 (100)	116 (100)	426 (100)	137 (100)	NA	NA
Chest in-drawing, n (%)	4 (1.3)	0 (0)	4 (.9)	1 (.7)	0.6(1)	1.0(1)
Rhonchi, n (%)	208 (67.1)	77 (66.4)	285 (66.9)	77 (56.2)	1.0(2)	0.03(2)
Stridor, n (%)	0 (0)	0 (0)	0 (0)	0 (0)	NA	NA
Crackles, n (%)	88 (28.4)	32 (27.6)	120 (28.2)	32 (23.4)	1.0(2)	0.3(2)
Treatment on presentation						
Antibiotics prescribed, n (%)	310 (100)	115 (99.1)	425 (99.8)	136 (99.3)	0.3(1)	0.5(1)
Other treatment prescribed, n (%)	310 (100)	115 (99.1)	425 (99.8)	137 (100)	0.3(1)	1.0(1)
Bronchodilator	182 (58.7)	66 (56.9)	248 (58.2)	75 (54.7)	0.8(2)	0.5(2)
Corticosteroids	28 (9.0)	14 (12.1)	42 (9.9)	13 (9.5)	0.5(2)	1.0(2)
Mucolytic agent	32 (10.3)	15 (12.9)	47 (11)	15 (10.9)	0.6(2)	1.0(2)
Cough syrup	223 (71.9)	79 (68.1)	302 (70.9)	92 (67.2)	0.5(2)	0.5(2)
Antipyretics	85 (27.4)	29 (25)	114 (26.8)	33 (24.1)	0.6(2)	0.6(2)
Antihistamines	30 (9.7)	13 (11.2)	43 (10.1)	23 (16.8)	0.8(2)	0.05(2)
Diagnosis (1st follow up)						
Upper respiratory infection	82 (26.5)	27 (23.3)	109 (25.6)	45 (32.9)	0.6(2)	0.1(2)
Lower respiratory infection	228 (73.5)	89 (76.7)	317 (74.4)	92 (67.1)	0.6(2)	0.1(2)
Bronchitis	146 (47.1)	56 (48.3)	202 (47.4)	60 (43.8)	0.9(2)	0.5(2)
Bronchiolitis	67 (21.6)	29 (25)	96 (22.5)	26 (19)	0.5(2)	0.4(2)
Rhino - pharyngitis	58 (18.7)	18 (15.5)	76 (17.8)	29 (21.2)	0.5(2)	0.5(2)
Pharyngitis	19 (6.1)	8 (6.9)	27 (6.3)	15 (10.9)	0.9(2)	0.1(2)
Asthma	9 (2.9)	3 (2.6)	12 (2.8)	5 (3.6)	1.0(1)	0.8(2)

Pneumonia	6 (1.9)	0 (0)	6 (1.4)	1 (.7)	0.2[1]	1.0[1]
Tonsillitis	5 (1.6)	1 (.9)	6 (1.4)	1 (.7)	1.0[1]	1.0[1]
Laryngotracheobronchitis	0 (0)	1 (.9)	1 (.2)	0 (0)	1.0[1]	1.0[1]
Outcome (after 1 week)						
Completely recovery	86 (27.7)	18 (15.5)	104 (24.4)	34 (24.8)	0.01[2]	1.0[2]
Partial recovery	190 (61.3)	82 (70.7)	272 (63.8)	89 (65)	0.09[2]	0.9[2]
Unchanged	18 (5.8)	10 (8.6)	28 (6.6)	8 (5.8)	0.4[2]	0.9[2]
Worsening	6 (1.9)	5 (4.3)	11 (2.6)	5 (3.6)	0.3[2]	0.7[2]
Admitted to hospital	6 (1.9)	1 (.9)	7 (1.6)	0 (0)	0.7[1]	0.2[1]
Unknown	4 (1.3)	0 (0)	4 (.9)	1 (.7)	1.0[1]	1.0[1]

P1 value was calculated between single detection and co-detection

P2 value was calculated between pathogen-negative-group and pathogen-positive-group

[1]: Fisher's Exact test; [2]: Chi-square test; [3]: Mann-Whitney test

4.4 Discussion

These two studies are the first to be conducted in Vietnam employing a multiplex polymerase chain reaction method to detect 14 respiratory viruses and 6 bacteria in outpatients with ARI as well as in healthy children aged from 0 to 15 years.

The major findings of this chapter can be summarised as follows:

- There was overall a good agreement between NPA and NTS in the detection of respiratory pathogens in ARI outpatients, although differences in the detection rates of some separate pathogens were recorded and loads were higher in NPA specimens.
- SP and Hin were detected at a very high frequency (SP in virtually all patients and healthy children) and thus were not included as a pathogen in the analysis of our study.
- Overall detection rate of respiratory pathogens in ARI outpatients was 75.6%, of which co-infections accounted for 20.6%. A viral pathogen accounted for 72.5% and bacterial pathogens for 7.3%. The most commonly detected virus was hRV while MP was the most frequently identified bacterium.
- No significant differences were found regarding pathogen loads between single detection and co-detection of all pathogens tested in ARI patients.

- RSV and MPV were associated with higher rates of clinical diagnosis of LRI, while no significant difference between patients with single- or multiple pathogens were recorded with respect to the rate of LRI.
- Patients infected with a single pathogen were more likely to report full recovery than those infected with mixed pathogens.
- Respiratory viruses and bacteria were detected at substantial rates in healthy children.
- The overall detection rate as well as identification rates of most individual pathogens were markedly higher in ARI patients than those in healthy children as expected. Flu A, RSV A/B, and BPp were frequently detected in ARI patients but not found in any healthy children.

4.4.1 *Streptococcus pneumoniae* and *Haemophilus influenzae* in ARI patients and healthy children

The results from our two studies on ARI patients as well as healthy children, which employed identical diagnostic tests (multiple PCR assays) to detect pathogens in both children with and without ARI, showed that the detection rates of SP were not significantly different between ARI patients, asymptomatic children and healthy children with mild respiratory symptoms. Hin was detected more frequently in healthy subjects than those with ARI. Among ARI patients, the prevalence of both bacteria was similar regardless of severity of ARI (URI or LRI) or the presence of other pathogens. Pneumonia, the clinical entity which is mainly associated with SP and Hin according to textbooks and many studies [250-253], constituted only a very small percentage (7/563 or 1.2%) of our study population. We also found other viruses or atypical bacteria coexisting in respiratory samples of these patients with pneumonia.

As regards bacterial load, the distributions (shape of histogram) of Cp-value of these two bacteria were similar between ARI patients and healthy children, but both SP and Hin

were found to have significantly higher loads in ARI patients compared to healthy children. Among healthy children, SP was found to have significantly higher loads in children with mild respiratory symptoms compared with asymptomatic children. A possible explanation is that in comparison with asymptomatic healthy children, patients with ARI and mild respiratory symptoms shed more SP and Hin as well as the mucosal cells and fluids, which are secreted from the nose or throat from where the samples were taken during infection/inflammation.

Although patients with high loads of SP more frequently had severe ARI (LRI), higher loads were also found more frequently in younger patients, particularly in 1-2 year old children. Results from Chapter 3 showed that the rate of LRI was significantly higher in patients less than-5 years old, particularly in less than-2 year olds. Therefore, it could be argued that age was the confounding factor in the correlation between rate of LRI and rate of SP with low Cp-values. Indeed, the association of bacterial load with LRI did not remain significant after using multiple logistic regression analysis.

The presence of other aetiologic agents also did not differ between patients with low loads of SP and those with high loads of this bacterium. Similarly with Hin, there were no differences in terms of severity of ARI, age, and the presence of other pathogens between patients infected with Hin at different percentiles of Cp values.

These findings were consistent regardless of whether the specimen processed was NTS or NPA. Overall there were no associations between high loads of either SP or Hin and the severity of ARI and the coexistence of other pathogens.

In summary, we made every effort to differentiate between carriage and true invasive infections of SP as well as Hin in ARI patients but we have had no evidence to support the pathogenic role of SP and Hin in patients with mild ARI despite the fact that the rate of SP detected in our study (about 98%) is much higher than the carriage rate of SP in healthy

children in a study in Hong Kong (55.7%) [254], in healthy orphan children in Poland (63.3%) [255], and in healthy children attending day-care centres in the Czech Republic (38.1%) [256], to cite a few examples. In the light of these findings, it was concluded that these two bacteria reside commensally in the respiratory tracts in children with and without disease (but at much higher frequency than anticipated). Therefore, in this chapter we did not include SP and Hin as causative bacteria accounting for mild respiratory infections in the study population.

PCR on respiratory swabs cannot distinguish between carriage and disease. For viral ARI diagnostics and surveillance, nose and throat swabs are considered the sampling methods of choice, whereas for diagnostics of (more severe) pneumonia caused by Hin or SP, Gram-staining and bacterial culture of representative purulent sputum, blood culture or urinary antigen tests (for SP) would be the testing method of choice, which were not performed for this study.

4.4.2 Comparison of NTS with NPA for identification of respiratory causative agents

The overall detection rates and Kappa scores indicated a moderate to substantial agreement between NPA and NTS for the whole analysis of all pathogens as well as three-fourths (15/20) of individual pathogens. Among the remaining five pathogens, three (Flu A, hRV and BoV) were detected more frequently in NPA, and two (PIV-3 and MP) were detected more frequently in NTS.

In a previous study of 309 two-month to 13 year-old hospitalised patients with ARI in Ho Chi Minh City, Vietnam [16], Do et al found that the detection rate with NTS was very similar to NPA for all 15 respiratory viruses using a multiplex RT-PCR. Based on these findings, and the fact that NTS is less invasive and causes less discomfort, we had decided to use NTS as the sampling method to detect respiratory pathogens in healthy children. Our current study in outpatients shows more pronounced differences in detection rates,

including for four important pathogens Flu A, hRV, PIV-3 and MP among which two (Flu A and MP) may have therapeutic implications.

There have been several other studies that have compared NPA and NTS or nasal swabs for detection of respiratory pathogens. A study conducted at Turku University Hospital, Finland in 1999 of 230 hospitalised children with URI comparing nasal swab specimens and NPA samples subjected to virus culture for detection of respiratory viruses concluded that, with the exception of RSV which was more prevalent in NPA samples, there was no significant difference in the detection rates of other viruses between the two sampling methods [257]. Another study conducted in 295 children (41% were in-patients) presenting with respiratory symptoms to Royal Children's Hospital, Brisbane, Australia in 2007 compared NTS and NPA for the detection of eight respiratory viruses by means of real-time polymerase chain reactions. This study indicated that, with the exception of Flu B, the sensitivity of NTS samples was equal to, or lower than, that for the NPA specimens. For the common major viruses in children, the sensitivity of NTS specimens was considered high enough, at more than 90%. Thus, NTS sample combined with real-time polymerase chain reaction was recommended by these Australian researchers to be the diagnostic test of choice in outpatient settings as it is very much less invasive than NPA [258].

Considering the individual pathogens, such as RSV, although our study revealed no significant difference between NPA and NTS regarding detection rates of RSV, the study in Finland mentioned above revealed that RSV had a significantly higher detection rate in NPA (97%) compared to that in nasal swabs (76%; P = 0.001) [257]. Likewise, a study in Guinea-Bissau during 1996 – 1998 analysing paired specimens from 635 children with LRI, at the outpatient clinic of a national paediatric hospital as well as the children's clinic at a local health centre, showed that the sensitivity of antigen detection of RSV was 27%-

31% higher in NPA than in NTS (McNemar's test P < 0.0001) [259]. With respect to Flu A, while this virus was more prevalent in NPA than in NTS in our study (9.1% versus 6.9%, p = 0.02), a population-based prospective study at the San Juan University Hospital, Spain in 264 adult patients with novel influenza A/H1N1 infections found that NTS, combined with Real-Time Reverse Transcriptase PCR for influenza A/H1N1 virus detection, gave a higher diagnostic yield than NPA [260].

When results from NTS and NPA samples were combined, the identification rate of one or more pathogens in ARI patients was significantly higher compared to NTS samples or NPA specimens alone. This finding was in line with the study in Spain which also indicated that the combination of NTS and NPA had a significantly higher sensitivity in identifying the novel influenza A/H1N1 2009 virus than did each method alone [260].

Despite the inconsistencies in detection rates of several individual pathogens between the two methods, our study was in line with other studies in indicating an overall acceptable agreement between NTS and NPA for the detection of respiratory pathogens. NTS has the advantage of being easier to perform, cheaper and less invasive than NPA. In the rest of this chapter, when presenting data on aetiology in ARI patients, we used the results from NTS samples in order to obtain representative comparisons with the results in healthy children.

4.4.3 **Viral and bacterial pathogens in ARI outpatients**

Among our 563 outpatients with ARI, at least one pathogen was detected in 75.6% of cases, of which single infections made up 55% and co-infections accounted for 20.6%. These findings were in agreement with recent studies among children with ARI in outpatients which also employed real-time RT-PCR to identify respiratory pathogens in children [36, 52] (Table 4-15). In Madagascar, the detection rate of at least one pathogen was 74.6% and the rates of single infection and co-infection were 47.3% and 27.3%,

respectively [36]. In the Netherlands, a study which included both in- and out-patients revealed that 73.4% were positive for at least one causative agent, 46.9% positive for one pathogen and 26.5% for multiple pathogens [52].

Similar results were also seen in studies among hospitalised patients with ARI [16, 51] (Table 4-15). In Do et al's study in Ho Chi Minh City, 72% of children with severe ARI were positive for one or more viruses and 20% positive for mixed pathogens [16]. Lower rates of overall detection as well as co-infection were recorded in Yoshida et al's study in Khanh Hoa, at 69% and 12.4%, respectively [51].

Many other studies in the literature aimed to identify respiratory pathogens in children with ARI and the detection rates of causative agents varied from 35% to 87% [1, 2, 4, 7, 22, 27-30] while rates of co-infection ranged from 0.3% to 22% [1, 2, 4, 27, 29]. Reasons for the wide disparities in detection rates in the literature included differences in clinical manifestations (URI or LRI), heterogeneity in ages of study participants (children aged 0-15 or aged below 5), differences in number of respiratory pathogens tested, and different laboratory tests for pathogen detection. Most studies in the literature obtained lower detection rates of single as well as mixed pathogens than in our study as they employed conventional methods such as cell culture and immunofluorescence assays [1, 4, 7, 22, 27-30] which are less sensitive than real time PCR. Moreover, aetiologic agents tested in these studies were limited to several respiratory viruses and did not include bacteria [1, 4, 7, 22, 27-30] compared to the 14 viruses and 6 bacteria in our study.

As regards separate causative agents in ARI patients in our study, hRV was the most commonly detected pathogen (27%-152/563), followed by EV (10.5%- 59/563). Previous experience from others [211, 261, 262] has shown that the hRV primers and probe used here may cross-react with EV RNA and thus may cause false positive hRV results and an overestimation of the detection rate of hRV. In fact the seasonality of these two viruses

was very different in our population, and there were only twenty-two cases of co-detection. If there was cross-reactivity, one would expect the EV Cp-value to be lower than the hRV Cp-value; this was only the case in a minority of these 22 and therefore we expect that the contribution of cross-reactivity to co-detection of EV and hRV would be small.

Similar rates of hRV were observed in the studies in Madagascar (20.5%) [36], in the Netherlands (24.1%) [52], and in Khanh Hoa, Vietnam (28%) [51] where hRV was also the most [36, 51] or the second most common pathogen [52]. As for RSVA/B, the detection rate of this virus (9.6%-54/563) in our study was comparable to the rate in outpatients in Madagascar (11.8%) [36] but much lower than among populations of exclusively or partially hospitalised patients in Khanh Hoa (23%) [51], the Netherlands (36.6%) [52], and Do et al's study (24%) [16], with the latter two studies identifying RSV as the most frequently detected pathogen in ARI patients. In general, hRV is detected most commonly at outpatient settings while RSV was the most frequently detected virus in hospitalised ARI patients.

As regards bacterial detection in our study, MP was identified most frequently, at 4.4%. This rate was much lower than that (16.2%) in Spuesens et al's study which also used real-time PCR to determine MP infection among 321 children with ARI attending Erasmus MC–Sophia Children's Hospital and the after-hours General Practitioners Cooperative in Rotterdam, the Netherlands from 2008 to 2011 [77]. This study also questioned the relevance of MP detection using PCR, as they found MP in high percentages (>20%) of asymptomatic children too.

Table 4-15 Studies on aetiology of ARI in children using real time PCR methods

Author; Year; Country	Laboratory test for pathogen detection	Number of pathogens tested	Participants	Study settings	Study period	Results		
						Any pathogen positive	Single infection	Co-infection
Hoffman et al, 2012, Madagascar, [36]	multiplex real-time RT-PCR	18 respiratory viruses and 2 atypical bacteria	295 outpatients with ARI between 2 to 59 months	Community hospital	2010-2011	74.6%	47.3%	27.3%
Huijskens et al, 2012, The Netherlands, [52]	RT-PCR	19 viruses and MP	177 outpatients and in-patients < 18 years with ARI	paediatric outpatient clinic and paediatric ward	2010	73.4%	46.9%	26.5%
Do et al, 2011, Vietnam, [16]	multiplex PCR	15 respiratory viruses	309 hospitalised children from 2 months to 13 years with ARI	referral hospital for infectious diseases	2004-2008	72%	-	20%
Yoshida et al, 2010, Khanh Hoa, Vietnam, [51]	multiplex RT-PCR	13 respiratory viruses	958 hospitalised patients below 5 with ARI	General hospital	2007-2008	69%	-	12.4%

4.4.4 Detection of respiratory pathogens and clinical manifestations of ARI

In our study, RSV and MPV were more prevalent in patients with LRI than in those with URI. For the other pathogens, there were no significant differences regarding detection rates of pathogens between LRI and URI. In Madagascar, hRV and PIV were also found, in addition to MPV, at higher rates in LRI than in URI. However, RSV and AdV were less common in LRI than in URI [36]. A study in hospitalised children with ARI in North-east Brazil showed that rates of single detection and co-infection of pathogens were similar regarding clinical manifestations as well as severe grades of ARI with the exception of

RSV, which was more common in severe disease and MP, which was associated with more severe cases of pneumonia [21].

As regards respiratory symptoms, our study showed that runny nose and nasal congestion were recorded more commonly in the pathogen-positive-group than in the pathogen-negative-group. Interestingly, the rate of sore throat in patients without pathogen was significantly higher than that in children infected with at least 1 causative agent. This may be explained by the fact that sore throat is a subjective symptom which is difficult to recognise in infants and young children. As a result, the true rate of this symptom could be underestimated, particularly in young children. In addition, patients in the pathogen-negative-group had a higher median age than those in the pathogen-positive-group. The proportion of patients with no pathogen detected was significantly higher in the over-5 group (Table 4-12), who in turn had a significantly higher rate of sore throat than the younger group. Therefore, age is a confounding factor in the correlation between pathogen-negative group and a higher frequency of sore throat.

Rhonchi were observed more frequently in the pathogen-positive-group than in the pathogen-negative-group. These findings were consistent with those in the study of Huijskens et al where patients in the pathogen-positive-group were younger than those in the pathogen-negative-group. Rhinorrhoea and wheezing were also observed more frequently in patients where respiratory pathogens were detected than in those without pathogen [52].

In the pathogen-positive-group, there were no significant differences regarding the proportion of URI as well as of LRI between the single-pathogen group and the multiple pathogen group although patients infected with more than one pathogen had a lower rate of complete recovery than those infected with single pathogens. There were several other studies which also reported no differences between single and multiple infections with

respect to disease severity [52, 263-265] whereas some studies demonstrated that mixed infections are associated with more severe disease [266, 267].

4.4.5 Virus and bacteria detections in healthy children

Between the two groups of 255 healthy children (asymptomatic vs. mildly symptomatic children), there was a significant difference regarding detection rates of the total number of respiratory pathogens, and the most frequently detected ones. In the asymptomatic group, despite the fact that we did not record any respiratory symptoms or clinical findings in all 216 children, almost 20% of cases were positive for one or more pathogens, of which more or less 4% were co-infection. According to Jansen et al, there are several explanations for this: symptomatic but subclinical infection, i.e. symptoms/signs are mild and not recognised/reported (this was clearly shown in our study: 39 patients reported having respiratory symptoms when asked, and many of these patients were detected with pathogens); incipient infection or at the period of incubation and symptoms had not yet developed (Flu); past infection, i.e. prolonged shedding (AdV) or PCR tests detecting remnants of recent infections; or asymptomatic carriage of these pathogens [268, 269].

Jansen et al, using the same multiplex PCR, demonstrated that 28% of 157 asymptomatic children coming to the outpatient ward at the Academic Medical Centre in Amsterdam were positive for one or more viruses [269]. Advani et al detected an even higher percentage (41.7%) of respiratory viruses in 158 children with no symptom of respiratory viral illness attending the Johns Hopkins Hospital Children's Centre in Maryland, USA [270].

For individual pathogens, Jansen [269] and Advani [270] also found hRV to be the most common virus, at 15% and 32% respectively, in their study among participants without respiratory symptoms. The overall detection rate of hRV in these studies was higher than in our study (10.6%). The difference may be due to the disparities in study settings and

age of study participants in the three studies. Our study included 0-15 year-old children while the other two studies enrolled only children up to 6 years [270] or younger than 3 [269].

4.4.6 Comparison of aetiology detection between ARI patients and healthy children

As expected, the overall detection rates of most individual pathogens were considerably higher in ARI patients than those in healthy children. This finding was expected and in line with other studies [269, 270]. Of note, there were several pathogens which were not detected in any healthy children, including Flu A, RSV A/B, and BPp. Other studies have also demonstrated that RSV is rarely detected in asymptomatic children [53, 57, 269] . This suggests that RSV should be considered as the causative agent of disease when detected in symptomatic subjects. Jansen et al observed that hRV, CoV and BoV were commonly identified in asymptomatic children and thus, assuming a causal connection between the detection of these viruses and clinical symptoms should be made with caution in symptomatic patients [269]. Our study supported these findings in the sense that hRV was frequently detected in the control group (13%), while CoV and BoV had similar detection rates in both symptomatic and asymptomatic subjects.

While our study found a significant difference regarding the detection of MP between ARI patients (4.4%) and healthy subjects (2.2%), Spuesens et al's study in the Netherlands showed that the detection of MP in symptomatic patients (16.2%) did not differ significantly from asymptomatic children (21.2%) although the prevalence of MP in both groups of patients in this study were much higher than that in our study. When bacterial loads of MP between symptomatic and asymptomatic groups were compared, there was an agreement between our study and Spuesens et al's study in showing similar distributions of MP loads in children with and without respiratory symptoms.

One of the limitations in this chapter is that there were differences between the two study populations regarding the number of children allocated into four age groups (Table 4-1): there were many more ARI children aged 2 to 5 years than healthy children, whereas healthy children aged over 5 significantly outnumbered ARI children in the same age group. This was because the healthy control group was established to serve as a control group for multiple studies on respiratory infection and therefore we aimed to have equal rates of participation (more or less 25%) among the 4 age groups, while in the study of ARI patients we did not. In addition, the two studies were not conducted simultaneously; hence, the healthy population was not an ideal control group. Another limitation is that hRV primers and probe used in our study may cross-react with EV RNA and thus may cause false positive hRV results and an overestimation of the detection rate of hRV. However, the contribution of cross-reactivity to co-detection of EV and hRV, as discussed above, was small.

In summary, this chapter has provided an in-depth analysis of respiratory pathogens detected in outpatient children with ARI and provided a comparison with those in healthy children in Ho Chi Minh City. The unexpectedly high detection rate of SP of almost 100% both in patients and healthy children makes PCR detection of this pathogen in swabs or NPA of no value for clinical decision making and thus should not be used for these purposes. SP pneumonia is diagnosed using traditional gram-staining and culture, blood culture or urinary antigen testing. This is similar for Hin (with the exception of urinary antigens). There have been studies that show that low Cp values in sputum samples of hospitalised patients with pneumonia may be predictive for causation of SP or Hin when detected, but we could not reproduce this reliably in swabs of patients with mild disease. In this setting, we conclude therefore, that molecular testing for SP and Hin is not useful and should be discouraged.

The high detection rate of viral pathogens in our study confirms results from other studies and once more suggests that antibiotic use for treatment of outpatient ARI in children is probably not appropriate in the majority of cases. This will be further discussed in the next chapters.

Chapter 5

Selection of resistant *Enterobacteriaceae* in the gut flora of children with acute respiratory infections presenting to outpatient clinics in Vietnam

5.1 Introduction

In Chapter 4 we have shown that viruses were the dominant aetiologic agents in outpatient children with ARI in southern Vietnam. This is in keeping with many other studies [1-4, 7, 16, 36, 52]. Despite this, in Chapter 3 we see that almost all outpatients with ARI in our study were prescribed antibiotics directed at bacterial pathogens.

In Vietnam, most antibiotics are available over the counter without prescription, leading to high levels of uncontrolled and unrestricted antibiotic use. A survey in Vietnam showed that 62% of children under five had received antibiotics during the previous month [96]. Such a massive (over)use of antibiotics will inevitably have consequences for the resistance rates among human pathogens and commensal flora, including in the human gut [271].

The human gut flora is the natural habitat for a large bacterial community, which includes both commensal and pathogenic bacteria. Antibiotic use may have a harmful effect by selecting resistant bacteria in faecal flora. Under antibiotic pressure, there will be a selection for both bacteria with spontaneous mutations causing resistance or bacteria which carry plasmids with resistance genes. Resistant bacteria, in turn, may transfer these resistance genes to other commensal or pathogenic bacteria through conjugation, transformation or transduction [178, 272-280], and person-to-person spread through faecal-oral transmission may occur.

In this chapter, we aimed to describe the consequences for the normal human gut flora of antibiotic use in children with ARI.

5.2 Methods and Materials:

The study population was derived from the study of 563 ARI outpatients presented in the previous chapters. Rectal swab specimens were collected in 1ml of normal saline (0.9%) on admission and at follow-up visits. After collection, swabs were serially diluted tenfold and plated on MacConkey agar plates and incubated at 37 °C overnight. The next day, the number of lactose fermenting *Enterobacteriaceae* (characterised as round smooth pink colonies) were assessed and the dilution rendering a colony count of between 20 and 200 was recorded. This dilution was made again from the original specimen and subsequently plated on nine MC plates (one without and eight with antibiotics) and incubated at 37 °C overnight. The next day, colonies on all plates were counted.

Colony counts were then log10 (n+1) transformed and displayed in graphs for each antibiotic tested with counts on MC plates without antibiotic on the x-axis and counts on MC plates with antibiotics on the y-axis. The differences between the fractions at day 0 and 7, and at day 0 and 28 were assessed using the Wilcoxon signed-rank test. The fraction of resistant *Enterobacteriaceae* for each antibiotic was calculated as the ratio of numbers of colonies on plates with and without antibiotics.

Details of the study design, materials and methods are described in Chapter 2.

5.3 Results

There were 563 and 544 rectal swabs collected on day 0 and day 7, respectively. An interim analysis of the first 100 patients showed a significant increase in the fraction of resistant *Enterobacteriaceae* to almost all tested antibiotics and therefore it was decided to collect an additional rectal swab sample on day 28 from a subset of patients to assess the duration of this effect. 35 rectal swabs were collected on day 28.

The resistance to eight antibiotics is shown in graphs which display the log transformed colony counts on MC plates with and without antibiotic for rectal swabs collected on day

0, day 7 and day 28. The differences in the fractions of resistant bacteria between faecal samples collected at different time points were calculated using Wilcoxon Signed Ranks tests and expressed as p values. The change in resistant fractions was considered statistically significant if the p value was under 0.05.

5.3.1 **Antibiotics used before and on the day of presentation**

Results from Chapter 3 showed that 561/563 (99.6%) ARI patients were prescribed antibiotics on the day of presentation. The most commonly used antibiotics were amoxicillin-clavulanic acid (45.6%), cefuroxime (22%), cefixime (11.4%), cefaclor (8.2%), erythromycin (3.7%), amoxicillin (3.0%), and cefpodoxime (2.3%), while cotrimoxazole accounted for only 0.4%.

Before coming to the hospital, 180/563 (32%) patients had used antibiotics within a month before presentation. Out of these 180 patients, 174 (96.7%) were given beta-lactam antibiotics, among which amoxicillin (with or without clavulanic acid), cefaclor, cefixime, and cefuroxime were the most commonly used one, at 42.2% (76/180), 22.8% (41/180), 13.3% (24/180), and 12.2% (22/180), respectively. Looking at more recent use of antibiotics before presentation, 125/563 (22.2%) patients had been given antibiotics within a week prior to presentation. Among these 125 patients, 120 (96%) used beta-lactam antibiotics, of which amoxicillin (with or without clavulanic acid) was the most frequently used (37.6% - 47/125), followed by cefaclor (28.8%-36/125), cefuroxime (13.6%-17/125), and cefixime (11.2%-14/125). In 41% of cases (231/563) the parents did not know whether the children received antibiotics or not, as they bought medications recommended by the staff at pharmacies or private clinics without prescription.

5.3.2 Proportion of patients with *Enterobacteriaceae* resistant to antibiotic classes on presentation and follow-up

Table 5-1 shows high proportions of patients in whose gut flora *Enterobacteriaceae* resistant to tested antibiotics were found. Meropenem was an exception with only four (0.7%) patients carrying resistant bacteria. The highest resistance rates were found for cotrimoxazole (93.2%), tetracycline (91.8%), and amoxicillin (91.1%), followed by amoxicillin-clavulanic acid (89.5%), ceftazidime (67.3%), gentamicin (59.1%), and ciprofloxacin (57.2%).

Seven days after presentation, significant increases were observed in the proportion of bacteria resistant to amoxicillin, amoxicillin-clavulanic acid, ceftazidime, gentamicin, and ciprofloxacin. On day 28, i.e. after withdrawing antibiotics, there were no significant changes in the resistant rates to these antibiotics compared to those on day 0.

Table 5-1 Proportion of children with ARI in whose gut flora *Enterobacteriaceae* resistant to different antibiotics were detected at different time points

Proportion of children with bacteria resistant to antibiotic	Day 0	Day 7	Day 28	P1 (Day 0 vs. Day 7) *	P2 (Day 0 vs. Day 28)*
Amoxicillin, n (%)	513/563 (91.1)	516/542 (95.2)	28/35 (80.0)	0.008	0.5
Amoxicillin- clavulanic acid, n (%)	504/563 (89.5)	513/541 (94.8)	28/35 (80.0)	0.001	1.0
Ceftazidime, n (%)	379/563 (67.3)	446/542 (82.3)	13/35 (37.1)	0.000	0.2
Ciprofloxacin, n (%)	322/563 (57.2)	361/542 (66.6)	20/35 (57.1)	0.000	0.2
Gentamicin, n (%)	333/563 (59.1)	377/542 (69.6)	19/35 (54.3)	0.000	0.3
Tetracycline, n (%)	516/562 (91.8)	498/542 (91.9)	29/35 (82.9)	1.0	0.5
Cotrimoxazole, n (%)	524/562 (93.2)	513/542 (94.6)	25/35 (71.4)	0.4	0.3
Meropenem, n (%)	4/562 (0.7)	2/542 (0.4)	0 (0)	0.7	NA

*McNemar test

5.3.3 Changes in resistant fractions of *Enterobacteriaceae* in gut flora after seven days of antibiotic treatment

Table 5-2 shows the changes in the resistant fractions of *Enterobacteriaceae* in the patient's gut flora to eight antibiotics one week after patients were seen at the outpatient

clinic for ARI. Highly significant increases in resistant fractions were found in 5/8 antibiotics (62.5%) including amoxicillin, amoxicillin-clavulanic acid, ceftazidime, gentamicin and ciprofloxacin (Wilcoxon Signed Ranks Test, p value = 0.000). No significant changes were observed for tetracycline, cotrimoxazole, and meropenem. In fact almost no *Enterobacteriaceae* resistant to meropenem were cultured.

Table 5-2 Changes in fractions of *Enterobacteriaceae* in gut flora resistant to different antibiotics on presentation at the outpatient clinic and on day 7 among children with ARI. Significant changes are proven by Wilcoxon Signed Ranks Test. Mean of resistant fraction is shown to illustrate the difference

Antibiotic	Mean of resistant fractions at presentation (before treatment)	Mean of resistant fractions after 7 days treatment	P1 value (*)	Significant change in resistant fractions
Amoxicillin	0.84	0.94	0.000	Yes
Amoxicillin-clavulanic acid	0.82	0.92	0.000	Yes
Ceftazidime	0.50	0.70	0.000	Yes
Ciprofloxacin	0.40	0.50	0.000	Yes
Gentamicin	0.41	0.52	0.000	Yes
Tetracycline	0.82	0.84	0.36	No
Cotrimoxazole	0.85	0.89	0.054	No
Meropenem	0.004	0.002	0.5	No

(*): Wilcoxon Signed Ranks Test

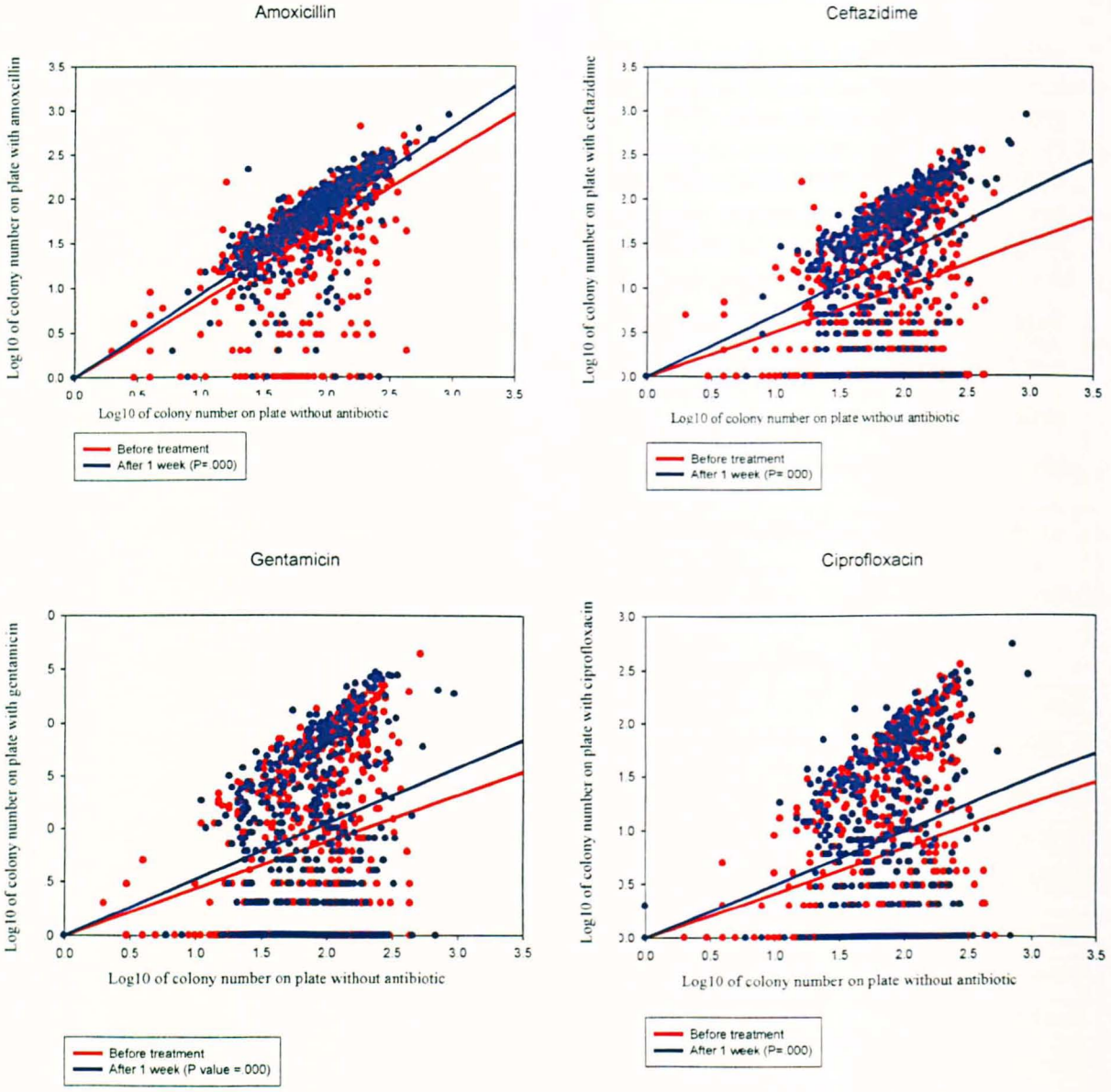

Figure 5-1 Comparison of colony counts of *Enterobacteriaceae* from gut flora of children with ARI when grown on plates with or without antibiotics between two time points: Day 0 and Day 7

Red line: regression of resistant fractions on Day 0

Blue line: regression of resistant fractions on Day 7

5.3.4 Changes in resistant fractions of *Enterobacteriaceae* in gut flora after 28 days

In the subset of 35 patients who came back for a second follow up, the resistant fractions of *Enterobacteriaceae* decreased (Table 5-3). Of note was the fact that the resistant fractions of *Enterobacteriaceae* to amoxicillin, ceftazidime and cotrimoxazole were lower on day 28 compared with those on day 0 although the differences were significant for only ceftazidime and cotrimoxazole.

Table 5-3 Changes in fractions of *Enterobacteriaceae* in gut flora resistant to different antibiotics on presentation at the outpatient clinic and on day 28 among children with ARI. Significant changes are proven by Wilcoxon Signed Ranks Test. Mean of resistant fraction is shown just to illustrate the difference

Drugs	Mean of resistant fractions before treatment	Mean of resistant fractions 28 days after treatment	P2 value (*)
Amoxicillin	0.84	0.79	0.06
Amoxicillin-clavulanic acid	0.82	0.82	0.87
Ceftazidime	0.50	0.29	0.005
Ciprofloxacin	0.40	0.44	0.1
Gentamicin	0.41	0.44	0.7
Tetracycline	0.82	0.83	0.28
Cotrimoxazole	0.85	0.68	0.02
Meropenem	0.004	0.000	0.3

(*):Wilcoxon Signed Ranks Test

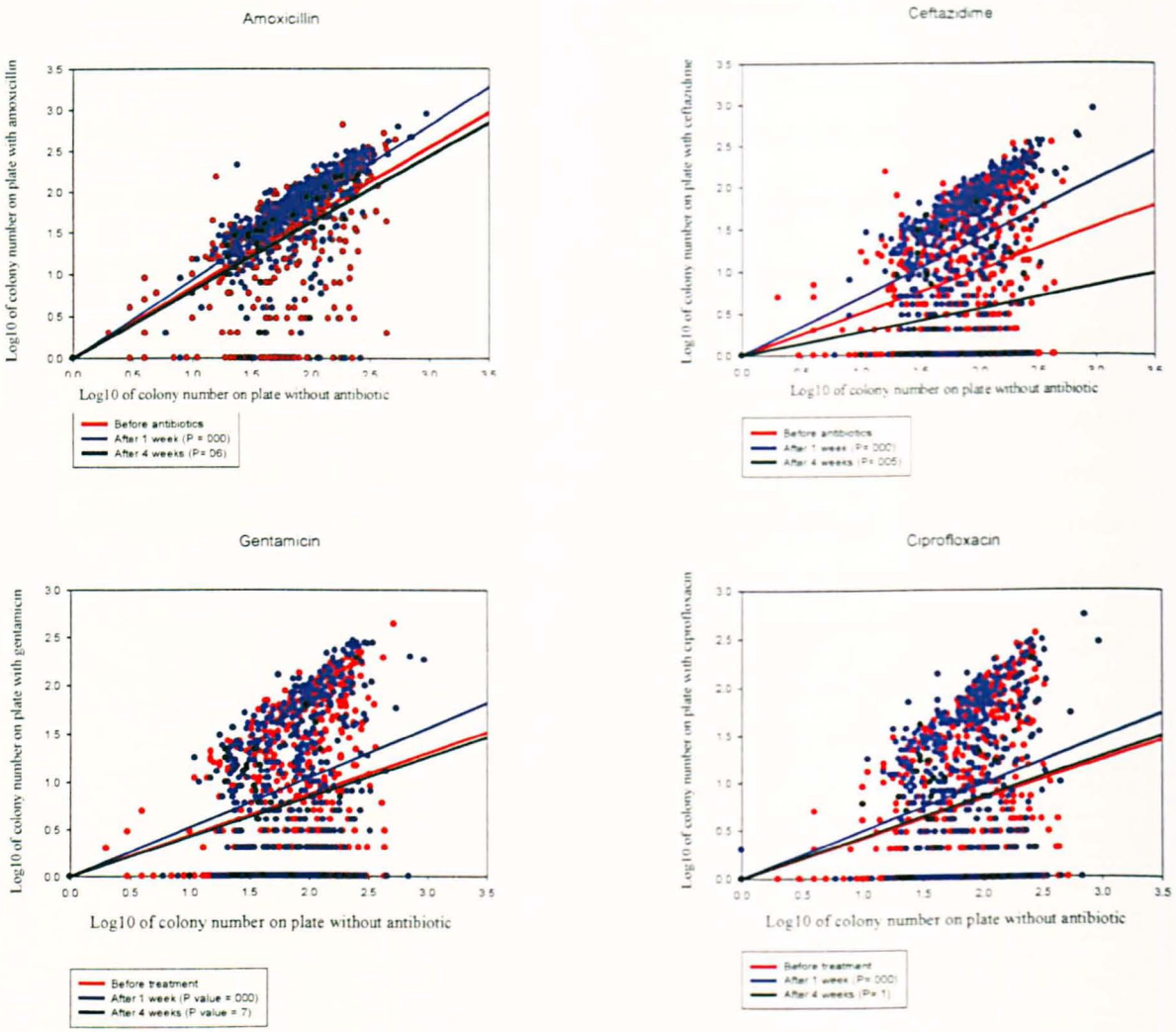

Figure 5-2 Comparison of colony counts of *Enterobacteriaceae* from gut flora of children with ARI when grown on plates with or without antibiotics between three time points: Day 0, Day 7 and Day 28

Red line: regression of resistant fractions on Day 0

Blue line: regression of resistant fractions on Day 7

Black line: regression of resistant fractions on Day 28

Among these 35 patients, there were seven (20%) who continued to use antibiotics during the period between day 7 and 28. There were no significant differences in the results for all 35 patients and the remaining 28 patients who did not use antibiotics during this period,

except for ceftazidime, where the difference in resistant fractions of *Enterobacteriaceae* between day 0 and 28 was no longer significant (Wilcoxon Signed Ranks Test, p value = 0.07) (Table 5-4).

Table 5-4 Changes in fractions of *Enterobacteriaceae* in gut flora resistant to different antibiotics on presentation at the outpatient clinic and on day 28 among the 28 children with ARI who stopped using antibiotics after day 7. Significant changes are proven by Wilcoxon Signed Ranks Test. Mean of resistant fraction is shown to illustrate the difference

Drugs	Mean of resistant fractions before treatment	Mean of resistant fractions 28 days after treatment	P2 value (*)
Amoxicillin	0.85	0.76	0.08
Amoxicillin-clavulanic acid	0.82	0.79	0.9
Ceftazidime	0.49	0.32	0.07
Ciprofloxacin	0.39	0.47	0.4
Gentamicin	0.41	0.44	0.7
Tetracycline	0.82	0.81	0.7
Cotrimoxazole	0.85	0.62	0.004
Meropenem	0.004	0.000	0.3

(*): Wilcoxon Signed Ranks Test

5.4 Discussion

5.4.1 Changes in resistant proportions and resistant fractions of *Enterobacteriaceae* after 7 days

On day 7, there were considerable increases in the proportion of patients with resistant *Enterobacteriaceae* in their gut flora to five of the eight antibiotics tested: amoxicillin; amoxicillin- clavulanic acid; ceftazidime; gentamicin; and ciprofloxacin. Similarly, highly significant increases were also observed in the resistant fractions of *Enterobacteriaceae* to these five antibiotics on day 7 compared to the day of presentation. This is explained by the effect of the antibiotic prescribed for almost all (99.6%) patients on the day of

presentation. Under antibiotic pressure, resistant bacteria were selected and became more prevalent in the patients' gut flora [182, 281].

An increase in resistance to amoxicillin or amoxicillin-clavulanic acid is easily understood as amoxicillin-clavulanic acid was the commonest antibiotic prescribed (in nearly 50% of patients) on day 0 in our study. As regards ceftazidime, which is an intravenous antibiotic that is used for in-patients, it belongs to the most frequently prescribed class of antibiotics (the cephalosporin subclass of beta-lactam antibiotics) in our study population: approximately 94 % of patients in our study were given beta-lactam antibiotics and these were all expected to cause selection of resistant organisms, for which we used ceftazidime as a marker. Gentamicin is a parenteral drug for hospitalised patients only and was not used by any of the patients in our study while oral ciprofloxacin is not indicated for use in ARI and was also not prescribed for any patients in our study (see Chapter 3). The increase in resistant fractions can be explained by the fact that the resistance to beta-lactams such as amoxicillin or ceftazidime are mediated by many mechanisms including efflux pumps, chromosomal AmpC and plasmid mediated beta lactamases, of which ESBL (Extended Spectrum Beta-Lactamases) carrying plasmids play an increasingly important part. Because ARI children in this study received no aminoglycosides or fluoroquinolone to select for resistance against these antibiotics, the co-selection of gentamicin or ciprofloxacin resistant bacteria makes it likely that ESBLs were the causative mechanism. ESBL carrying plasmids are known to co-carry a number of other genes, among which are genes encoding resistance to aminoglycosides (as gentamicin) [118-120] and genes (qnr and aac(6_)-Ib-cr) which are responsible for resistance to fluoroquinolones including ciprofloxacin [120-127]. Plasmid-mediated quinolone resistance determinants such as qnr and aac(6_)-Ib-cr were detected at a high prevalence among *Enterobacteriaceae* in commensal flora of both hospitalised patients and non-

hospitalised healthy individuals in Ho Chi Minh City [282]. In a subset of 300 ARI patients in our study (Appendix I), Le Thi Minh Vien et al investigated the co-selection of fluoroquinolone resistance genes in patients' stool samples by quantifying *qnr* genes before and 7 days after non-fluoroquinolone antibiotic administration, and found a significant increase in the quantity of the *qnr*B and *qnr*S genes on day 7 compared with day 0. This finding highlights a correlation between the use of non-fluoroquinolone antibiotics (such as beta-lactams) and increasing resistance among gut flora, in this case an increased prevalence of qnr genes [283], proven in other studies [120-127], to be carried on plasmids containing other resistance genes, particularly those encoding resistance to beta-lactams such as ESBL genes.

There was also an increase in the fraction of bacteria resistant to cotrimoxazole, although this was not statistically significant ($p = 0.054$). During recent decades, cotrimoxazole has been rarely used in children with ARI. This is because the reported resistance rates for most bacterial pathogens associated with pneumonia are high and severe adverse events, particularly skin reactions including Steven Johnson Syndrome, discourage physicians from prescribing this drug. There can be cross-resistance to amoxicillin-clavulanic acid or other beta-lactams and cotrimoxazole mediated by transmissible plasmids [119]. Enne and colleagues showed that sulphonamide resistance genes are plasmid-borne and genetically linked to other resistance genes. Due to this genetic linkage, the rate of sulphonamide resistance among *E.coli* remained high in the UK, despite a 97% reduction in usage [197]. This can also explain the high levels of resistance to cotrimoxazole in our ARI patients, for whom amoxicillin-clavulanic acid and other beta-lactams were the most frequently prescribed medications on presentation.

There were no changes in the proportion of strains resistant to tetracycline. Tetracycline is almost no longer used in children, particularly in the treatment of ARI. Tetracycline is not

recommended for use in children under 8 because of side effects such as stomach pain or upset, changes in skin colour and particularly permanent staining of teeth. As a result, there was no patient using tetracycline in our study.

Meropenem is a carbapenem antibiotic which maintains activity against ESBL-producing *Enterobacteriaceae*. Almost no resistance to meropenem was detected in both faecal samples on day 0 and day 7, and as a result, no changes between the two time points were found.

There have been several studies about the impact of antibiotic consumption on human intestinal flora but no studies exploring the effect of antibiotics prescribed in children with mild ARI not requiring hospital admission on the resistance of human gut flora. Most of the previous studies were performed in adult healthy volunteers with small sample sizes and focused on the changes in the number of colonizing bacteria and the resistance of bacteria to only the antibiotic studied. They did not assess changes in the fraction of resistant bacteria in gut flora to other commonly used antibiotics. In a study by Brismar et al, beta-lactamase activity was assessed and the number of amoxicillin-resistant *E.coli*, *Klebsiella* and *Enterobacter* spp. increased in ten healthy volunteers after using oral amoxicillin for 7 days [206]. Another study conducted by Floor and colleagues evaluated the effect of amoxicillin on the intestinal flora of 80 patients with bronchitis and detected amoxicillin-resistant *E. coli* isolates in 35% of patients [284]. Likewise, Edlund et al found an overgrowth of newly amoxicillin-resistant *Enterobacteriaceae* in all 10 healthy volunteers who were prescribed amoxicillin orally for 7-10 days [285]. Stark and colleagues found selection of resistant *Enterobacteriaceae* and increased beta-lactamase concentrations in the faecal samples of 14 patients during amoxicillin plus omeprazole treatment for *Helicobacter pylori* infections [286]. In Lambert-Zechovsky et al's study, an increase in ampicillin-resistant *E. coli* and overgrowth of *Klebsiella* spp. were observed in

gut microflora of 7 hospitalised children who received oral amoxicillin-clavulanic acid [207].

As regards cephalosporins, Nord et al found no new colonization with cefaclor-resistant bacteria in the intestine after giving cefaclor to 10 volunteers for 7 days [287]. Similarly, in Finegold et al's study, intestinal flora did not change significantly after six volunteers were given cefaclor at 750mg per day for 14 days [209]. For cefuroxime and cefpodoxime, Edlund et al studied 10 volunteers for each antibiotic but did not find new colonisation with resistant *Enterobacteriaceae* after the volunteers used one of these two agents for 7-10 days [285]. With respect to cefixime, Finegold et al found no development of resistance to this antibiotic in the gut flora of 6 volunteers given 400mg cefixime daily for 14 days [209].

5.4.2 **Restoration of gut flora after withdrawing antibiotics**

Resistance-associated mutations in the bacterial genome may be associated with a fitness cost [182, 190, 281, 288, 289]. Likewise, plasmids containing drug resistance genes can impose biological costs on the host bacteria leading to reduced growth of bacteria [182, 200, 201, 290]. As a result, susceptible bacteria usually out-compete resistant bacteria when there is no selective pressure of antibiotics. This can be seen in our study from the fact that after four weeks, complete restoration to the level at presentation before antibiotic prescribing was observed in case of amoxicillin, amoxicillin-clavulanic acid, gentamicin and ciprofloxacin.

Interestingly, the resistant fractions to amoxicillin, ceftazidime and cotrimoxazole of day 28 samples were even lower than in admission samples. This may be due to the fact that 32% (180/563) of patients were already using antibiotics on enrolment, and among these 180 patients, 96.7% were using beta-lactam antibiotics. Figure 5-3, Figure 5-4 and Figure 5-5 show that the resistant fractions of *Enterobacteriaceae* in gut flora to amoxicillin,

ceftazidime and cotrimoxazole in patients using antibiotics before admission were significantly higher than in those not using antibiotic (Mann-Whitney test, p value = 0.003, p value = 0.000 and p value = 0.04, respectively). As a result, when samples were collected on day 0, selection of resistant bacteria was already ongoing. Consequently, when antibiotic pressure was removed, intestinal flora restored to its original baseline with a lower resistant fraction than observed on day 0.

When we excluded 7 patients who continued to use antibiotics after day 7 and measured the resistant fractions of gut flora in the remaining 28 patients who did not use any antibiotic after day 7, the results were almost unchanged with the exception of ceftazidime, to which the resistant fraction of *Enterobacteriaceae* remained lower on day 28 than on day 0, but this difference was not significant. The explanation may be that the sample size of 28 patients was not large enough to reveal a statistically significant difference.

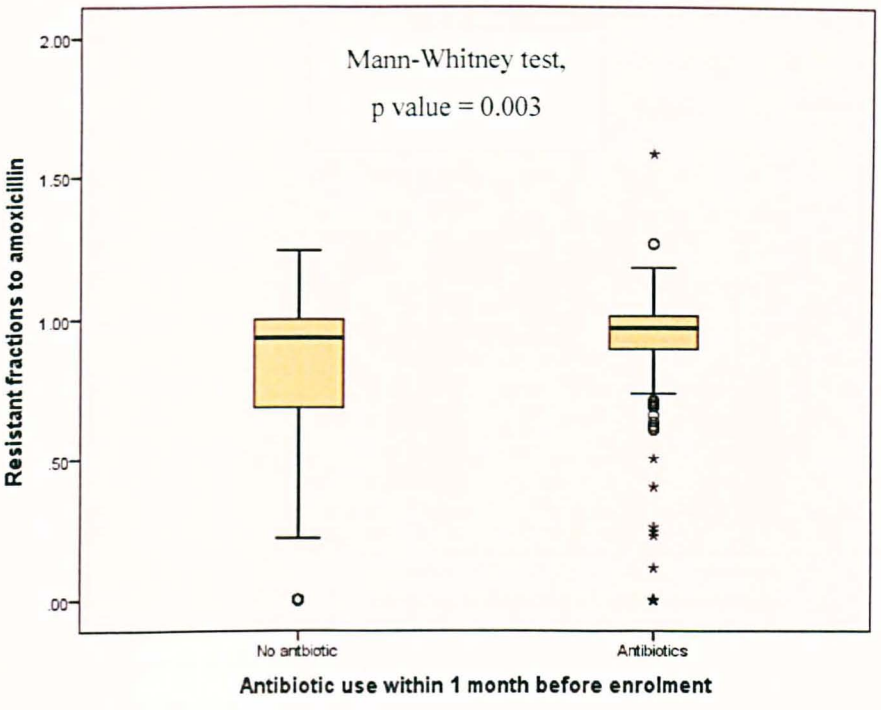

Figure 5-3 Resistant fractions of *Enterobacteriaceae* in gut flora to amoxicillin in 180 patients who received antibiotics within 1 month before enrolment and in 114 patients who did not.

Boxes: represents resistant fractions of 50% of cases with the lines inside indicating the median of resistant fractions. Small circles (o) and asterisks (★): outliers. Whiskers: protruding lines going out from the smallest resistant fraction (median minus 1.5 IQR) to the largest resistant fraction (median plus 1.5 IQR) (excluding outliers).

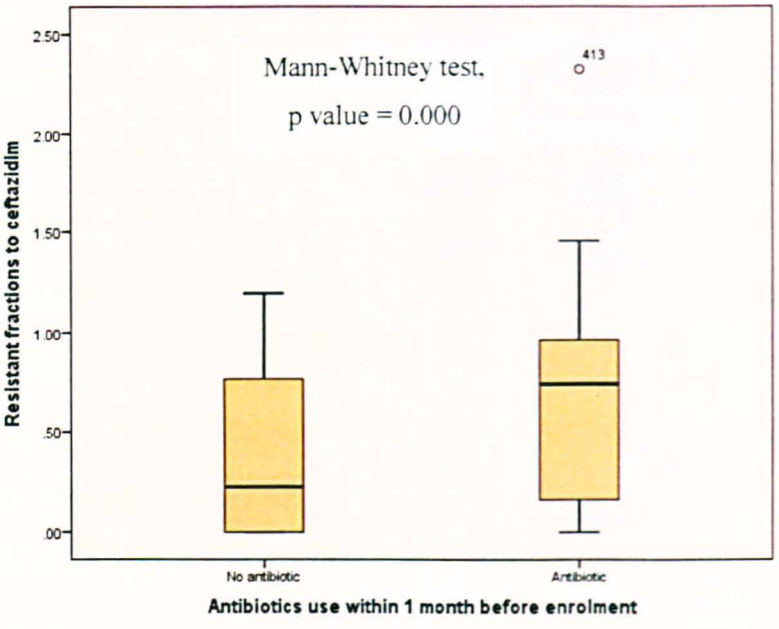

Figure 5-4 Resistant fractions of *Enterobacteriaceae* in gut flora to ceftazidime in 180 patients who received antibiotics within 1 month before enrolment and in 114 patients who did not

Boxes: represents resistant fractions of 50% of cases with the lines inside indicating the median of resistant fractions. Small circle (o): outliers. Whiskers: protruding lines going out from the smallest resistant fraction (median minus 1.5 IQR) to the largest resistant fraction (median plus 1.5 IQR) (excluding outliers).

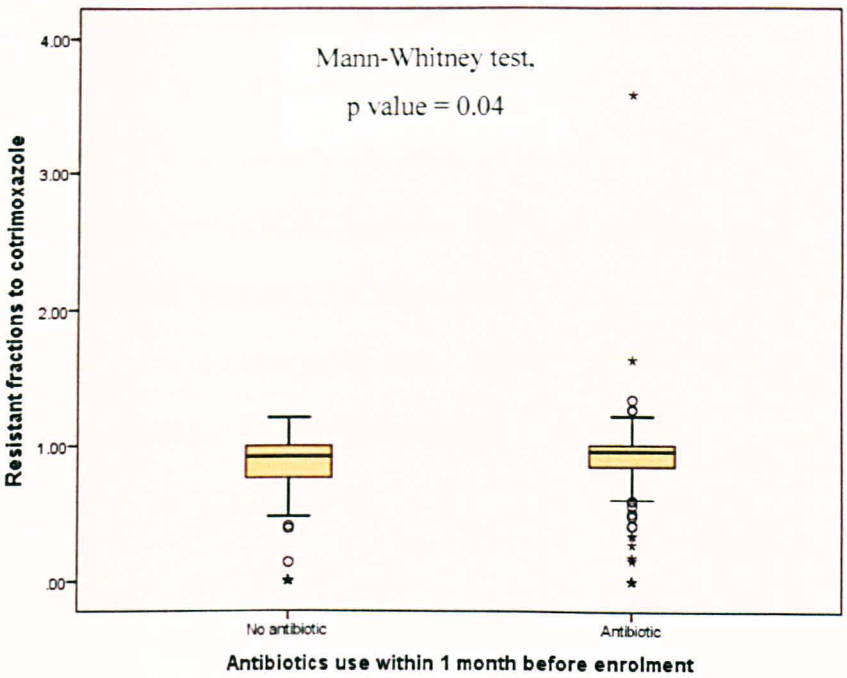

Figure 5-5 Resistant fractions of *Enterobacteriaceae* in gut flora to cotrimoxazole in 180 patients who received antibiotics within 1 month before enrolment and in 114 patients who did not

Boxes: represents resistant fractions of 50% of cases with the lines inside indicating the median of resistant fractions. Small circles (o) and asterisks (★): outliers. Whiskers: protruding lines going out from the smallest resistant fraction (median minus 1.5 IQR) to the largest resistant fraction (median plus 1.5 IQR) (excluding outliers).

Short-term effects of antibiotics on human intestinal flora were also found in many other studies. In a study conducted in the US in 2007 [291], healthy adults were recruited to collect stool specimens before, during, and 4 weeks after a short course of ciprofloxacin. One-third of the bacterial taxa in the gut was affected and reduced by ciprofloxacin treatment. However, the composition of the flora returned closely to the pre-treatment status by 4 weeks after the end of treatment. This resilience of human gut bacteria was

also observed in De La Cochetiere et al's study in which six healthy volunteers were given oral amoxicillin for 5 days and changes in dominant faecal microbiota were followed up. Within 30 days following antibiotic treatment, the gut flora returned and reached an average similarity of 88% to the original status on Day 0 [292].

Many recent studies also point out that the restoration of human gut flora is not complete, in other words, antibiotics not only have a short-term impact but also have a long-term effect on the selection of resistance within intestinal microflora. It is known that the acquisition of resistance usually comes with a fitness cost and hence, a reduction or removal in the use of antibiotics would result in selection of fit susceptible bacteria and a reduction of resistant bacteria. Nevertheless, additional fitness-compensatory mutations have helped reduce the biological costs for resistant bacteria allowing them to compete successfully with sensitive strains in an antibiotic-free environment leading to the persistence of several resistant genes for years [182, 184, 185, 189, 198, 199]. Similar phenomena have been observed for plasmid encoded antibiotic resistance. Although plasmids carrying resistance genes come with a biological cost to their bacterial hosts, plasmids have evolved mechanisms to ensure their successful 'transmission' to daughter cells. Compensatory mutations of the bacterial host genome reducing the fitness costs of plasmid replication have also been reported [200-202].

Jernberg et al reported a significant and persistent rise for up to two years in the levels of specific macrolide resistance genes in stool samples of healthy subjects after a 7-day clindamycin oral therapy [293]. Sjolund et al's study showed a persistence of resistant enterococci for up to three years in human intestinal microflora after short courses of clarithromycin for *Helicobacter pylori* eradication [294]. The long-term persistence of resistance genes among the human gut flora explains the very high rate of patients who carried resistant bacteria to almost all antibiotics tested on the day of presentation,

particularly for the high prevalence of resistance to cotrimoxazole and tetracycline which today are rarely used, especially in children.

This chapter describes the impact of antibiotic use on the selection of resistant *Enterobacteriaceae* in human gut flora. Although this effect seems only mildly hazardous for the individual patient, because of the restoration of gut flora after withdrawing antibiotics, given the magnitude of antibiotic use in Vietnam this will cause selection and transmission of resistant bacteria at a population level, which is highly undesirable from a public health perspective. If there is no intervention to address the uncontrolled and indiscriminate use of antibiotics, particularly in children with mild ARI as discussed in previous chapters, it may eventually lead to not only transient changes but also persistent resistance in human gut flora with consequences for treatment options when patients develop an infection caused by these bacteria.

Chapter 6

Assessment of antibiotic use at enrolment by parent interviews and high performance liquid chromatography of urine samples from children with ARI

6.1 Introduction

Despite legislation, in Vietnam most antibiotics (old and new, narrow and broad spectrum) are easily purchased over-the-counter at pharmacies without prescriptions, leading to high, uncontrolled and indiscriminate antibiotic use [95, 96, 295]. Among the 563 ARI patients in our study, 41% did not know whether they had received antibiotics or not within 2 days prior to hospital presentation as they bought medications from pharmacies or private clinics without prescriptions. These medications are usually sold without package inserts or additional explanation of what they are or their mechanism of action. This causes difficulties for physicians in choosing antibiotic treatment, particularly in the case of severe bacterial infections if patients do not know with certainty which medication they have used before or are still using. Hence, obtaining a reliable detailed history of medication taken during the current episode of illness including antibiotics plays a significant role in the course of any treatment.

In this chapter, we aimed to identify the presence of 6 different oral beta-lactam antibiotics in the urine of children with ARI presenting to the outpatient clinic of Children's Hospital 1. Urine was collected from the children on the day of enrolment to assess the pre-enrolment antibiotic use and the level of agreement between patient's history and measurements.

6.2 Methods and Materials:

The study population in this chapter was derived from the same 563 ARI outpatients presented in the previous chapters. Urine specimens were collected on enrolment in order to identify whether six different beta-lactam antibiotics (cephalexin, cefadroxil, cefaclor, cefixime, amoxicillin, and cefuroxime) had been used prior to presentation. These 6 beta-lactam antibiotics were selected because they are excreted mainly in the urine and therefore can be detected by High Perfomance Liquid Chromatography (HPLC), and because they are the most commonly used antibiotics in the community (Appendix A). Details of the study design, materials and methods are described in Chapter 2.

6.3 Results:

From 563 patients, 553 (98.2%) urine samples were collected. Collection from the other 10 patients (1.8%) was not feasible in time for daily transfer of study samples to OUCRU for storage.

6.3.1 HPLC method

We developed an HPLC method that focused on detection of the six beta-lactam antibiotics and differentiation of these antibiotics from any interfering substances commonly present in urine samples. The resulting HPLC method had a high sensitivity and specificity for these drugs, and was validated in accordance with the bioanalytical method validation guidelines as developed by the Food and Drug Administration (FDA) [224] and the European Medicines Agency [225]. The method detail is outlined in Chapter 2.

6.3.1.1 Specificity

The chromatograms of the six most frequently used antibiotics in urine samples are shown in Figure 6-1. The analytes were well defined and were separated from matrix

contaminants, with a good symmetrical shape at the respective retention times. Interfering compounds from urine were not found to co-elute with either of the antibiotics. The positive samples of patient 03AV-009 (amoxicillin), 03AV-209 (cephalexin, cefixime), 03AV-254 (cephadroxil, cefaclor) and 03AV-355 (cefuroxime) are presented in the same figures. One of the most important criteria to identify compounds when using HPLC is to compare the spectrum obtained in tested samples with a reference spectrum. The comparison of two spectra gives a similarity index (SI). When two spectra are obtained from the same compound, the closer to 1 the SI value is, the more similar the two spectra are considered to be. Two spectra are considered to be identical when the SI value is close to 1. Figure 6-2 presents the similarity indices of spectra of antibiotics which were detected in urine samples of patients mentioned above compared to those in solutions (reference spectra).

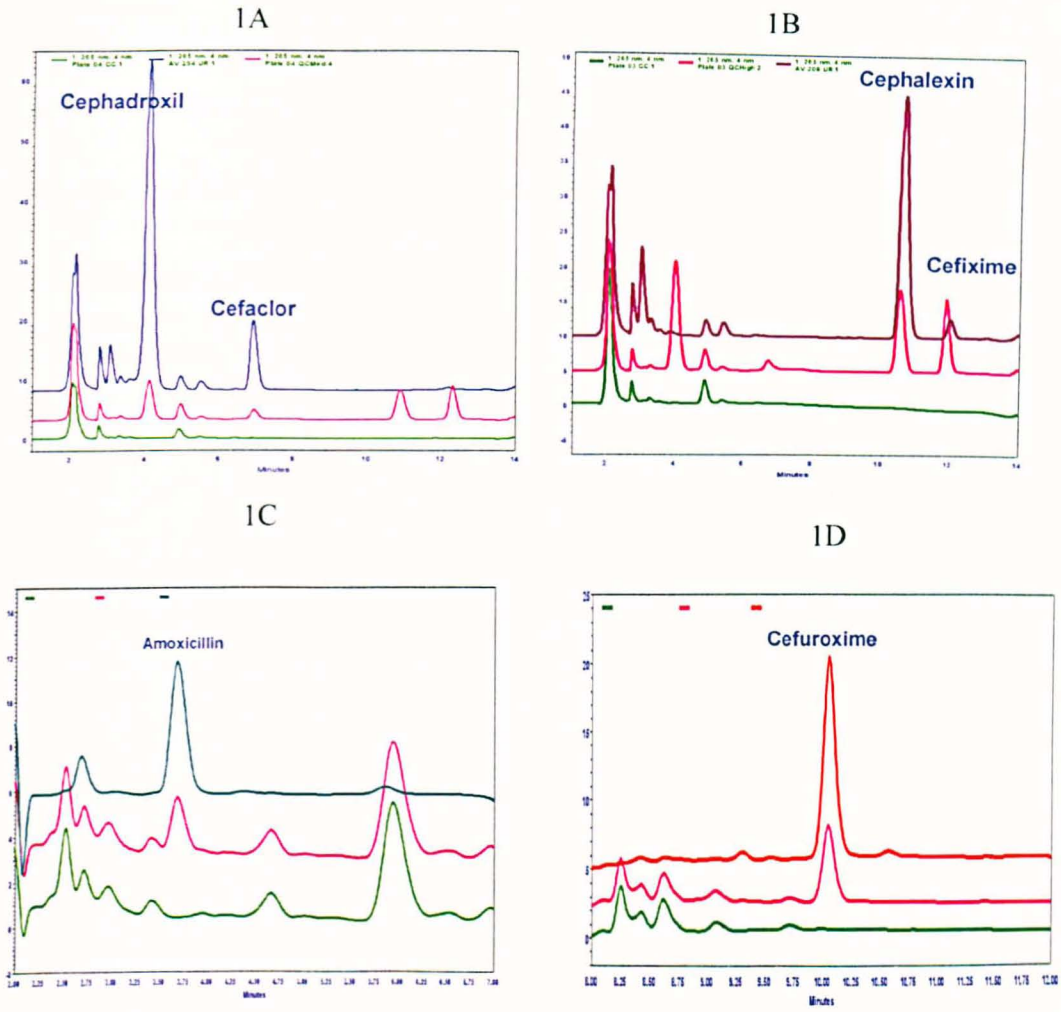

Figure 6-1 Chromatograms of positive antibiotics in urine samples of patients compared to blank urine and spiked urine samples.

1A: patient 03AV-254 (blue); 1B: 03AV-209 (brown); 1C: 03AV-009 (dark green) and 1D: 03AV-355 (red).

Pink line: Spiked urine with cefadroxil, cephalexin, cefaclor, cefixime (1A, 1B) or with amoxicillin and cefuroxime (1C, 1D).

Green line: Blank urine.

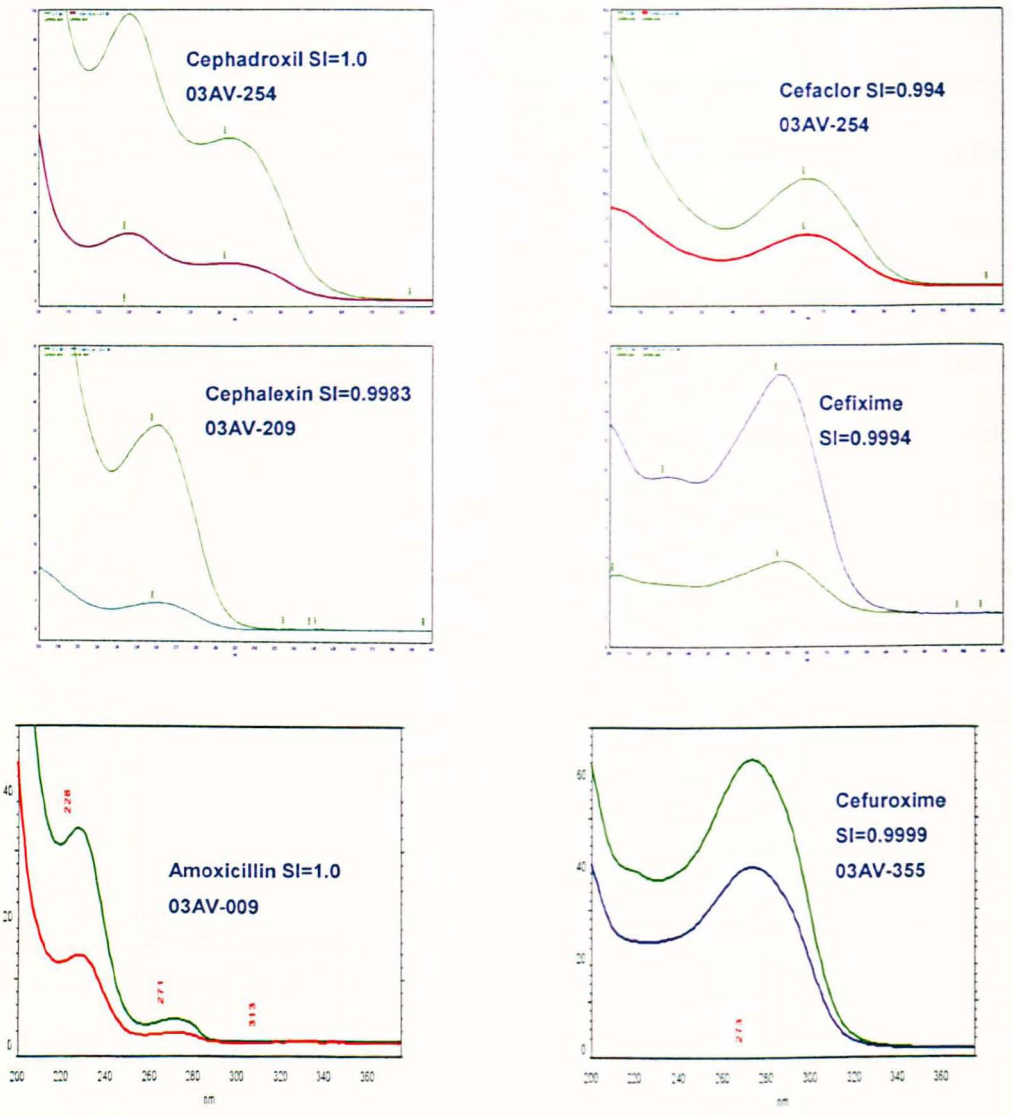

Figure 6-2 Similarity index between spectra of antibiotics tested in patients' urine and reference spectra

Green line: UV spectra of antibiotics detected in urine samples of patients.

Other colours: reference spectra.

6.3.1.2 Limit of detection (LOD) and lower limit of quantification (LLOQ)

After HPLC analysis of spiked urine samples containing cephalexin, cefadroxil, cefaclor and cefixime, the lowest concentration of medications that the HPLC procedure can

distinguish from background noise (LOD) was determined at 0.08 μg/mL, and the lowest amount of antibiotics in the samples that can be quantified (LLOQ) was 0.3 μg/mL.

As regards amoxicillin and cefuroxime, LLOQ was identified at 0.2 μg/mL while LOD at 0.1μg/mL (amoxicillin) and 0.05 μg/mL (cefuroxime).

Table 6-1 LOD and LLOQ in comparison with MIC for *Enterobacteriaceae* of tested antibiotics

Antibiotic	MIC for *Enterobacteriaceae* (*)	LOD	LLOQ
Amoxicillin	8μg/ml	0.1μg/mL	0.2 μg/mL
Cephalexin	16μg/ml	0.08 μg/mL	0.3 μg/mL
Cefadroxil	16μg/ml	0.08 μg/mL	0.3 μg/mL
Cefaclor	8μg/ml	0.08 μg/mL	0.3 μg/mL
Cefuroxime	4μg/ml	0.05 μg/mL	0.2 μg/mL
Cefixime	1μg/ml	0.08 μg/mL	0.3 μg/mL

(*): Source: Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing twenty-third informational supplement. M100-S23. January 2013 [296])

6.3.1.3 Linearity

Linearity of calibration consisted of 8 points (including blank urine) and was determined in the range of 0, 0.3, 0.6, 2, 6, 12, 20, and 30μg/mL (for cephalexin, cefadroxil, cefaclor and cefixime) and 0, 0.2, 0.5, 1, 2, 5, 10, and 20μg/mL (for amoxicillin and cefuroxime). The correlation coefficients (r^2) were > 0.99 for the calibration curves of all 6 antibiotics tested.

6.3.1.4 Precision and accuracy

Intra-day and inter-day precision and accuracy of HPLC method developed for identification of 6 beta-lactam antibiotics in urine samples are shown in Table 6-2. The coefficients of variation were less than 15% in all quality control levels for 6 antibiotics.

Table 6-2 Measurement of accuracy and precision in intra-day and inter-day analytical runs

Concentration levels of antibiotics	Nominal concentration (μg/mL)	Intra-day analytical runs			Inter-day analytical runs		
		n	*Precision (%)*	*Accuracy (%)*	*n*	*Precision (%)*	*Accuracy (%)*
Cefadroxil							
Low	0.5	4	2.1	104.4	20	14.7	109.9
Medium	10	4	2.1	92.9	20	12.6	102.5
High	25	4	8.0	102.9	20	8.2	100.6
Cefaclor							
Low	0.5	4	2.9	100.2	12	9.28	102.78
Medium	10	4	5.8	101.7	12	14.05	97.49
High	25	4	3.9	100.1	12	10.71	99.17
Cephalexin							
Low	0.5	4	2.9	100.2	20	11.8	102.8
Medium	10	4	4.2	90.8	20	13.5	101.2
High	25	4	3.7	97.71	20	8.6	98.2
Cefixime							
Low	0.5	4	3.05	101.4	20	10.8	103.7
Medium	10	4	5.33	95.3	20	12.2	101.3
High	25	4	3.28	105.6	20	7.7	100.9
Amoxicillin							
Low	0.4	5	7.6	93.4	25	10.4	92.3
Medium	4	5	5.3	103.6	25	6.8	102.3
High	16	5	2.4	88.1	25	4.6	89.9
Cefuroxime							
Low	0.4	5	5.0	87.4	25	8.7	85.1
Medium	4	5	4.3	89.1	25	4.1	87.4
High	16	5	1.9	86.4	25	4.2	87.3

6.3.2 Identification of antibiotics in the patient's urine

Table 6-3 shows that almost one-third (32.2% - 178/553) of urine samples were positive for at least one of the antibiotics tested. Two and three beta-lactams were found in 14 (2.5%) and 4 (0.7%) patients' urine, respectively. Cefixime was detected at the highest rate (54/553- 9.8%), followed by amoxicillin (52/553-9.4%), cefadroxil (34/553-6.1%), cefaclor (28/553-5.1%), and cephalexin (22/553-4.0%), while cefuroxime was the least commonly detected antibiotic, at 1.8% (10/553).

In urine specimens from 67.8% (375/553) of patients none of the six antibiotics tested were detected.

Table 6-3 Detection rates of antibiotics in 553 patients' urine on the day of enrolment

Determination of antibiotics in patient's urine	Number (total number=553)	Rate (%)
At least 1 antibiotic detected	178	32.2
1 antibiotic	160	28.9
2 antibiotics	14	2.5
3 antibiotics	4	0.7
Cefixime	54	9.8
Amoxicillin (with or without clavulanic acid)	52	9.4
Cefadroxil	34	6.1
Cefaclor	28	5.1
Cephalexin	22	4.0
Cefuroxime	10	1.8
Negative (below the level of detection)	375	67.8

6.3.3 Antibiotic use during the two days before enrolment identified by patient interviews

When interviewing parents on the day of enrolment, for 123/553 (22.2%) patients we recorded the use of at least one antibiotic during the two days prior to enrolment. Among these 123 patients, there were eight whose parents said they had used antibiotics which

were not among the six beta-lactam antibiotics tested. Among the remaining 115 cases (20.8%-115/553) who had used at least one beta-lactam antibiotic, there were 111/553 (20.1%) and 4/553 (0.7%) patients using one or two antibiotics, respectively. Amoxicillin was the most frequently used antibiotic, at 8.7% (48/553), followed by cefaclor (6.7%-37/553), cefuroxime (2.9%-16/553), and cefixime (2.5%-14/553) while cefadroxil and cephalexin were the two medications that patients used least, at 0.5% (3/553) and 0.2% (1/553), respectively (Table 6-4).

Table 6-4 Antibiotic use within 2 days before admission as identified by patient interviews

Antibiotic use within 2 days before admission	Number (total number=553)	Rate (%)
At least 1 antibiotic used	123	22.2
1 antibiotic	119	21.5
2 antibiotics	4	0.7
Amoxicillin (with or without clavulanic acid)	48	8.7
Cefaclor	37	6.7
Cefuroxime	16	2.9
Cefixime	14	2.5
Cefadroxil	3	0.5
Cephalexin	1	0.2
Other antibiotics (cefpodoxime, cefdinir, erythromycin, spiramycin, oxacillin)	8	0.2
No antibiotic	206	37.3%
Unknown	224	40.5%

6.3.4 Comparison of antibiotic identification between urine tests and parent interviews

Among 329 patients whose parents answered "Yes" or "No" when asked about the history of antibiotic use in the two days before enrolment, there was agreement between the results of urine tests and parent interviews in 237 patients (72%-237/329), of which 18.2%

(60/329) were found positive and 53.8% (177/329) negative in both urine test and parent interviews, respectively (Table 6-5). Among 60 patients who were positive in both urine tests and parent interviews, there were 50 cases (83.3%) (including 22 cases for amoxicillin, 3 for cefadroxil, 1 for cephalexin, 12 for cefaclor, 4 for cefuroxime, and 8 for cefixime) in which generic names of antibiotics identified by urine tests were in line with those obtained through parent interviews (Table 6-6). For the remaining ten (16.7%) children, the antibiotic generic name detected in urine by HPLC tests was different from the name reported by parents or legal guardians (Table 6-7). At the same time, among 29/329 (8.8%) children, antibiotics were detected in urine while parents had answered "no" when asked about antibiotic use in the previous two days. In contrast, there were 63 (19.2% - 63/329) children in whose urine we could not detect any antibiotics while parents had answered "yes" when asked about use of any antibiotics (Table 6-5). 89% (56/63) of these had used the antibiotics we tested for in the previous two days. Of these children, 7/63 had used other antibiotics (erythromycin, spiramycin, cefpodoxime, oxacillin).

In order to assess the level of agreement between the HPLC method and patient interviews in identifying antibiotic use, a Kappa score was calculated and its value of 0.37 indicated a low agreement between the two methods (Table 6-5).

Among 224 cases with unknown history of antibiotic use, we detected at least one antibiotic in 89 patients' urine (39.7% - 89/224) by HPLC.

Table 6-5 Agreement regarding antibiotic identification between urine tests and patient interviews in 329 patients who knew the status of antibiotic use before enrolment

Consistency of antibiotic identification between urine tests and patient interviews N= 329					
Yes			No		
Positive in both urine tests and interviews	Negative in both urine tests and interviews	Total	Positive in urine tests, "negative" in interviews	Negative in urine tests, "positive" in interviews	Total
60 (18.2%)	177 (53.8%)	237 (72%)	29 (8.8%)	63 (19.2%)	92 (28%)

Measurement of agreement - Kappa score	Value	P value
	0.37	0.000

Table 6-6 Comparison of generic names of antibiotics as reported by parents/legal guardians and as identified by HPLC tests

* The denominator of all percentages is 553

a. Amoxicillin

	Parent interview	**Yes**	**No**	**Unknown**	**Total**
HPLC test					
Positive, n (%)*		22 (4)	9 (1.6)	21 (3.8)	52 (9.4)
Negative, n (%)*		26 (4.7)	272 (49.2)	203 (36.7)	501(90.6)
Total		48 (8.7)	281 (50.8)	224 (40.5)	553 (100)

b. Cefadroxil

	Parent interview	**Yes**	**No**	**Unknown**	**Total**
HPLC test					
Positive, n (%)*		3 (0.5)	9 (1.6)	22 (4)	34 (6.1)
Negative, n (%)*		0 (0)	317 (57.3)	202 (36.5)	519 (93.9)
Total		3 (0.5)	326 (59)	224 (40.5)	553 (100)

c. Cephalexin

	Parent interview	**Yes**	**No**	**Unknown**	**Total**
HPLC test					
Positive, n (%)*		1 (0.2)	7 (1.3)	14 (2.5)	22 (4)
Negative, n (%)*		0 (0)	321 (58)	210 (38)	531 (96)
Total		1 (0.2)	328 (59.3)	224 (40.5)	553 (100)

d. Cefaclor

	Parent interview	Yes	No	Unknown	Total
HPLC test					
Positive, n (%)*		12 (2.2)	6 (1.1)	10 (1.8)	28 (5.1)
Negative, n (%)*		25 (4.5)	286 (51.7)	214 (38.7)	525 (94.9)
Total		37 (6.7)	292 (52.8)	224 (40.5)	553 (100)

e. Cefuroxime

	Parent interview	Yes	No	Unknown	Total
HPLC test					
Positive, n (%)*		4 (0.7)	3 (0.5)	3 (0.5)	10 (1.8)
Negative, n (%)*		12 (2.2)	310 (56.1)	221 (40)	543 (98.2)
Total		16 (2.9)	313 (56.6)	224 (40.5)	553 (100)

f. Cefixime

	Parent interview	Yes	No	Unknown	Total
HPLC test					
Positive, n (%)*		8 (1.4)	13 (2.4)	33 (6.0)	54 (9.8)
Negative, n (%)*		6 (1.1)	302 (54.6)	191 (34.5)	499 (90.2)
Total		14 (2.5)	315 (57)	224 (40.5)	553 (100)

Table 6-7 Discrepancies in antibiotic identification between urine HPLC tests and parent interviews

Patient's study number	Antibiotic determination by HPLC tests	Antibiotic use by parent interviews
AV-011	Cefixim	Cefdinir
AV-028	Cefadroxil	Cefixime
AV-035	Cephalexin	Amoxicillin- clavulanic acid
AV-173	Amoxicillin	Cefuroxime
AV-252	Cefadroxil, cephalexin	Cefaclor
AV-338	Cefixime	Amoxicillin- clavulanic acid
AV-373	Cefadroxil	Cefaclor
AV-386	Cefadroxil	Amoxicillin- clavulanic acid
AV-400	Amoxicillin	Cefaclor
AV-504	Cefaclor	Cefuroxime

6.4 Discussion

6.4.1 Development of HPLC method to identify the presence of beta-lactam antibiotics in the urine

In this chapter, we aimed to develop an HPLC assay for detection of six beta-lactam antibiotics in patients' urine. The method showed high sensitivity and specificity, and helped to detect and differentiate our six targeted antibiotics in the presence of intrinsic interferences in urine samples with reasonable LLOQ (0.3μg/mL for cephalexin, cefadroxil, cefaclor, and cefixime; 0.2μg/mL for amoxicillin and cefuroxime). A partial validation procedure was carried out, and the results met all acceptance criteria of bioanalytical method validation guidelines laid down by the Food and Drug Administration and European Medicines Agency.

In brief, the results of method validation indicated that our HPLC assay method was highly sensitive and specific, and hence, may be appropriate for qualitative determination of antibiotics in the human urine.

6.4.2 Antibiotic determination in patients' urines by HPLC assay method

Using HPLC, we determined that there were 32.2% of patients whose urines were positive for at least one of six beta-lactam antibiotics tested. This means that about one-third of patients had used at least 1 antibiotic before the urine samples were collected at the time of enrolment. This figure may not include patients who had taken their last dosage more than 24 hours before we collected their urine, due to the fact that, in all of six beta-lactam antibiotics tested, 50% of the doses are excreted in the urine within 2-6 hours after oral administration and almost all of the medications are excreted in urine and eliminated from the body within 24 hours (data from adults) [297]. For instance, about 50%-70% of amoxicillin, which was recorded as the most commonly used antibiotic before enrolment, is excreted in the urine within 2-6 hours after oral administration. The remainder is

eliminated in the next few hours until the concentration of medication remains at trace levels in the urine and below the level of detection (LOD) by HPLC after more or less 1 day [297].

Table 6-8 Serum half life and elimination in the urine of six antibiotics [297].

Antibiotic	Serum half life	Amount of antibiotic excreted in urine within 8-12 hours after oral administration
Cephalexin	2.5 hours	75-90%
Cefixime	2.4 – 4 hours	Unknown
Cefaclor	0.6 – 1 hour	50-85% (within 8 hours)
Amoxicillin	0.7 – 1.4 hours	43-80% (within 6-8 hours)
Cefadroxil	1.1 – 2 hours	70-90% (6-9 hours)
Cefuroxime	1.4 – 1.9 hours	Unknown

Since not all antibiotics were tested by means of HPLC, we can extrapolate that more than one-third of patients with mild ARI in our study had been given antibiotics within 1-2 days prior to enrolment. This finding was similar to that of a bi-centre study conducted in Vellore and Chandigarh, India which also indicated that 31% of 64 children with febrile illness were demonstrated by HPLC to have antibiotics in the urine [298]. There were 3.2% (18/553) of patients in whose urine we detected two or three antibiotics. This can be explained by the fact that patients may have received more than one antibiotic when they visited a pharmacy or they may have visited multiple medical clinics and/or pharmacies at almost the same time.

The results obtained by HPLC tests conclusively demonstrated widespread overuse of antibiotics among outpatients before coming to medical settings. It is noteworthy that cefixime, a new generation cephalosporin, was the most common antibiotic given to children with mild ARI in the community. This inappropriate use of antibiotics in the

community may account for increasing levels of antimicrobial resistance, particularly to newly developed antibiotics.

6.4.3 Comparison of HPLC method with parent interviews in identification of antibiotic use within 2 days prior to admission

The overall detection of the six beta-lactam antibiotics as determined by urine HPLC tests was approximately 12% higher than recorded during parent interviews (32.2% versus 20.8%). Our validated HPLC method provides more accurate (and higher) rates of antibiotic use than subjective information given by parents, but failed to detect antibiotics in 56 urine samples from patients who claimed to have used the six drugs we tested for. Furthermore, at least one antibiotic was identified in almost 40% (89/224) of patients who recorded 'don't know' when asked whether they had used antibiotics or not within two days prior to presentation.

The Kappa score of 0.37 showed a low level of agreement between the HPLC assay method and patient interviews in assessment of antibiotic use. In 72% of cases we found consistency of results (positive and negative) between urine HPLC measurements or parent interviews regarding antibiotic use. In the remaining 28% of patients in whom there were discrepancies - either we detected antibiotics in urine while parents did not record use, or we did not detect antibiotics by HPLC while parents reported the use of antibiotics - these discrepancies were most likely accounted for by faulty reporting by parents. Other reasons for a negative HPLC result may be poor-quality medications, non-compliance with prescriptions or having taken the last dose too long ago to be able to detect traces in urine. The study in Ba Vi pointed out that almost 50% of caregivers gave their children antibiotics without professional consultations, i.e. either self-treatment (17%) or seeking advice and/or treatment at drug stores (33%) [96]. This study also demonstrates that only 13% of caregivers had correct knowledge about antibiotic use in ARI. These findings

highlight the likelihood of incorrect use and/or poor dose compliance in which caregivers may either increase doses in the hope of helping their children recover more quickly or, otherwise reduce doses to prevent side effects of medications. As a result, inadequate doses lead to lower antibiotic concentration in urine than expected and thus, urine tests may be negative while parents reported the use of antibiotics. In addition, counterfeit medications are considered as a big issue in Vietnam. According to statistical data of the Ministry of Health in 2008, Vietnam ranked second in Southeast Asia regarding fake medications, and 0.21% of total domestic and imported drugs (most of which were antibiotics) were found to be counterfeit medications and withdrawn from Vietnam's pharmaceutical market [299, 300]. The popularity of low-quality medications may contribute to a negative result for the detection of antibiotics in the urine when parent interviews had reported antibiotic use. Determining the presence of medications in the urine by HPLC assay method has its own drawbacks. The window of opportunity for each antibiotic varies from antibiotic to antibiotic. Although the serum half-life of an antibiotic is known, the precise time point for complete elimination of that antibiotic from the body is not. It is likely that this will be within 24 hours after the last dose. The frequency of children's urination from the last dosage of antibiotics taken to urine sample collection also has a great impact on the concentration and detection of medication in the urine samples: the more frequently a child urinates before sample collection, the lower the probability for detection of antibiotics in the urine specimens. Therefore, in some cases, parents may confirm the use of antibiotics 1-2 days prior to urine collection while HPLC test reveals negative results.

The differences between generic names of antibiotics detected in urine and those reported by parents in ten patients (Table 6-7) may be due to misidentification of the names of antibiotics by parents because the specificity of the HPLC methods that we developed is

high enough to make us confident about the accuracy of urine test results. In addition, there may be many different trade names for a single generic drug name purchased in Vietnam's pharmaceutical market. For instance, amoxicillin-clavulanic acid is being sold under more than ten different trade names. Likewise, cefuroxime, cefaclor or cefixime are manufactured by more than fifteen pharmaceutical companies and marketed using various trade names (Table 6-9).

Table 6-9 Different trade names of common oral antibiotics sold in Vietnam

Generic name	Trade names
Amoxicillin-clavulanic acid	Augmentin; amoxiclav, augbactam, augtifar, curam; comoxiclav, clamax, clava, enhacine, klamentin, clavamox, clavucillin
Cefuroxime	Cefuroxim, quincef, zaniat, zinat, kalerox, travicef, cadicefur, haginat, eurozetroxi, cefurobiotic, zalrinat, rigocef, zyroxim, midancef, doroxim, cefusan, cefurovix
Cefixime	Cefixim, fisec, mecefix, pentacef, ifixim, trifix, acicef, effixent, hafixim, hwafix, lufixime, orenko, okenxim, cipcef, fix, dahaxim
Cefaclor	Cefaclor, ceclor, faclor, imeclor, traclor, ilclor, cadicefaclor, kefcin, colorfast, ceplor, cidiclor, dahaclor, keflor, vercef, mekocefaclor

In summary, with its very high sensitivity, specificity and selectivity which were validated as discussed above, the HPLC method has provided accurate data on antibiotic use before enrolment in addition to data from parent interviews. The figure of 32.2% of patients in whom we detected antibiotics in urine reflects a truly high rate of antibiotic use within 1-2 days before hospital presentation and points to a widespread overuse of antibiotics in the community, as almost 80% of patients using antibiotics were infected with viruses (see Chapter 4). Moreover, compared to parent interviews where we asked about antibiotic use

in the previous 1-2 days, the positive HPLC results in 32.2% of patients reflects antibiotic use within an estimated 12-24 hours (but definitely less than 2 days) before taking the urine specimen. These results highlight the significance of continuing medical education for medical staff and pharmacists on rational prescribing of antimicrobials. More importantly, existing legislation preventing pharmacies from selling antibiotics without prescriptions should be implemented.

Chapter 7

General discussion, conclusions, future work and recommendations

In this thesis, I have addressed the following research questions:

1. What is the magnitude and spectrum of antibiotic use and other treatments in children with mild ARI in an outpatient setting in Ho Chi Minh City, Vietnam?
2. How does the antibiotic use reported by patients/parents/legal guardians correlate with HPLC-assessed antibiotics in urine?
3. Which viruses and bacteria can be detected in respiratory swabs of patients with mild ARI as compared to a control group of healthy children in Ho Chi Minh City, Vietnam?
4. What is the magnitude of inappropriate antibiotic use in outpatients with ARI in Ho Chi Minh City, Vietnam?
5. What is the effect of antibiotic use on the antibiotic resistance of the patients' normal gut flora?

7.1 Antibiotic and other treatments for mild ARI in children in Ho Chi Minh City

Among 563 children with ARI in our study, there were 72.6% (409/563) of cases diagnosed as LRI including bronchitis (262/563-46.5%), bronchiolitis (122/563-21.7%), asthma exacerbation (17/563-3.0%), and pneumonia (7/563-1.2%). The remaining (154/563-27.4%) were classified as URI consisting of nasopharyngitis (105/563-18.7%), pharyngitis (42/563-7.5%), and tonsillitis (7/563-1.2%).

According to the newest evidence-based guidelines and textbooks [99-102, 235, 236], pneumonia and streptococcal pharyngitis are the two main indications for prescription of antibiotics. Pneumonia accounted for a very small proportion (1.2%) of children in our study. The prevalence of streptococcal pharyngitis is very low in children under 5 years of age [236], who made up almost 95% of children with ARI in our study. For other ARI

such as nasopharyngitis, bronchitis or bronchiolitis, antibiotics have not proven effective and therefore are not recommended unless there are bacterial super-infections, which occur in only around 5%-8% of children with URI [301, 302]. In our study, we did not record the occurrence of or suspicion for bacterial super-infections. In short, there was a very low number of ARI children in our study who had a clinical indication for antibiotic prescription.

In practice, there were 99.6% (561/563) of children with mild ARI in our study prescribed antibiotics on the day of presentation. This is a remarkable figure reflecting the low threshold for prescription and usage of antimicrobials in an outpatient setting. Moreover, the most commonly used antibiotics in our ARI patients, particularly in URI, were broad spectrum/new generation beta-lactam drugs such as amoxicillin-clavulanic acid, cefuroxime, cefixime or cefaclor, which are not recommended for ARIs according to current evidence-based guidelines [12, 99, 234, 236, 241]. It is noteworthy that these prescriptions were made by experienced paediatricians who were well-educated regarding the management of these illnesses in children, particularly in ARI. The antibiotic overuse can be explained by several possible reasons: lack of accurate rapid diagnostic tests to discriminate between viral and bacterial pathogens, or of haematological/biochemical markers (like CRP or white blood cell count) to indicate bacterial super infections, which makes it difficult for physicians to rule out bacterial causative pathogens and thereby refuse to prescribe antibiotics. In addition, several studies have attributed high rates of antibiotic prescription to parental expectations/pressure, which is another important factor that affects physicians' decisions [303-305]. In fact, many patients come to CH1, a tertiary referral hospital for paediatrics, after they have previously visited other clinics (or pharmacies) but did not recover, and now expect their children to be cured. The very high volume of patients (around 5,000 outpatients per day) and resulting time constraints

(doctors at CH1 have around 2-3 minutes per patient for examination and prescription) at the outpatient ward of CH1 may be other reasons which leave doctors here with very little time for examination to make appropriate judgements on antibiotic use. Incentives from pharmaceutical companies given to doctors who prescribe specific antibiotics could provide another explanation, although this was not specifically explored.

Apart from antibiotics, other medications prescribed for ARI in children were also not in accordance with treatment guidelines. About 80% of patients with bronchitis or bronchiolitis were given oral bronchodilators, which have been shown to have no benefit for reducing the need for admission, shortening illness duration at home (in bronchiolitis), or in relieving cough (in bronchitis). Likewise, around 20% of cases were prescribed with OTC cough products, which have been proven unsafe for younger children and are not recommended by WHO.

In brief, what physicians prescribed for children with mild ARI in clinical practice in CH1 almost always did not conform to standard treatment guidelines for mild cases of ARI, which do not indicate use of antibiotics or other medications such as corticosteroids, oral bronchodilators, antihistamines, and OTC cough syrups.

7.2 Antibiotic use before admission as reported by patients/parents/legal guardians and as assessed by HPLC assay method in urine samples

The rate of pre-treatment with antibiotics within 2 days prior to presentation was determined through patient/parent/legal guardian interviews to be approximately 21% of 563 ARI patients. At the same time, this rate as detected by an HPLC assay in the urine was about 32%. The Kappa score of 0.37 indicated a low agreement between the HPLC assay and patient interviews in determination of antibiotic use. The HPLC assay may provide a more accurate and a higher detection rate of antibiotic use than the information given by parents. Since not all antibiotics were tested by HPLC, we can confirm that more

than one- third of patients with mild ARI in our study had been given antibiotics within 1-2 days prior to admission, and this figure would be higher if we took into account patients who had used antibiotics more than 2 days before hospital presentation (around 10% of 563 ARI patients). This high rate of prior treatment with antibiotics reflects the over-the-counter (OTC) availability and widespread use of these drugs without prescription. In Vietnam, people have free access to all kinds of antibiotics although there is legislation banning antibiotic purchase without prescriptions. OTC sale of antibiotics is extremely common in Vietnam. This was described in a study in Ba Vi province, in northern Vietnam, which showed that most antibiotics given to children with ARI by caregivers were obtained without consulting a doctor [95]. A study conducted by Nguyen et al found that for children with mild ARI, caregivers often self-treated or consulted drug sellers before consulting doctors at private clinics or public hospitals [96].

7.3 Viral and bacterial respiratory aetiologies in healthy children and patients with mild ARI in Ho Chi Minh City

By employing a multiplex polymerase chain reaction (PCR) to detect 14 respiratory viruses and 6 bacteria, we found an overall detection rate of respiratory viruses and atypical bacteria in ARI outpatients of 75.6%, of which a viral aetiology accounted for 72.5% and (atypical) bacterial pathogens for 7.3%. These findings help to firmly demonstrate that respiratory aetiologies in children with ARI in Ho Chi Minh City were similar to those in other countries worldwide in the sense that viruses were identified as the predominant pathogenic agents. In healthy children, respiratory viruses and bacteria were also detected at substantial rates (23.1%), although the overall detection rate as well as identification rates and relative viral loads of most individual pathogens were markedly lower than those in ARI patients.

In both healthy subjects and ARI outpatients, SP and Hin were detected at very high frequencies, making PCR detections of these pathogens in swabs or NPA of no value for clinical decision-making. These bacteria were found as commensals in the upper respiratory tracts and not as the causes of disease.

7.4 Appropriateness of antibiotic use in children with mild ARI

Based on which pathogens were detected and kinds of medications prescribed, we were able to classify antibiotic use as inappropriate use, appropriate use. inappropriate choice, appropriate choice or unknown. We used the clinical diagnosis and (RT-) PCR results for the classification.

Both SP and Hin were identified at very high frequencies in the respiratory tract of children with and without symptoms of ARI. For bacterial load, the distributions (shape of histogram) of Cp-values for these two bacteria were similar among ARI patients and healthy children. Among ARI patients, we found that there were no associations between the higher loads of SP and Hin with the severity of ARI and the coexistence of other pathogens. Therefore, it was concluded that these two bacteria reside commensally in the respiratory tracts in children with and without disease, and we did not include SP and Hin as causative bacteria accounting for respiratory infections in our study population. If antibiotics were prescribed for patients only infected with SP or Hin in our study, this would be considered as inappropriate, except for patients with a clinical diagnosis of pneumonia, which is primarily caused by SP and Hin according to textbooks and many studies [250-253].

Therefore, the inappropriate use of antibiotics in our study was determined in patients who were not clinically diagnosed with pneumonia and whose respiratory samples were positive for at least one of 14 viruses and negative for all 6 bacteria tested by multiplex PCR. In contrast, antibiotic use would be categorised as appropriate if at least 1 of 6

atypical bacteria was detected in patients' respiratory samples or if the clinical diagnosis was pneumonia. The appropriate choice of antibiotic was defined as patient prescribed antibiotics with the correct spectra to cover the atypical bacteria tested, otherwise it would be considered as an inappropriate choice. Patients whose (RT-) PCR results for viruses and bacteria were negative were classified under the "unknown group" since not all respiratory pathogens were tested in our study and there may have been patients who were infected with pathogens which did not belong to the panel of pathogens tested in our study.

Given the definitions above, inappropriate use of antibiotics in our study was 67.7% (380/561). In contrast, 8.0% (45/561) was categorised as appropriate use of antibiotics. However, among these 45 patients, there were only 3 patients (6.6%-3/45) who were given macrolides (erythromycin or azithromycin), which are effective against atypical bacteria [306, 307]. These cases were classified as appropriate choice of antibiotics. The remaining 42 cases (93.4%) were categorised as inappropriate choice of antibiotics, such as beta-lactam antibiotics, which are inactive against atypical bacteria.

There were 137/561 (24.4%) patients categorised under the "unknown group", i.e. we could not verify the appropriateness of antibiotic use because PCR tests for viruses and bacteria were all negative.

There are many other studies which have described over- prescription of antibiotics in children with ARI [240, 308-313] (table 7-1). However, most of these studies assessed the inappropriateness of antibiotic use based on presumed viral respiratory diagnoses, not on the results of aetiologic tests. Presumed viral respiratory infections are acute URI, common cold (nasopharyngitis), or bronchitis while pneumonia, otitis media, and sinusitis are presumed to have bacterial origins. However, pneumonia may be viral (like influenza) and not all URI or bronchitis cases are caused by viruses. This can be seen from the fact

that among 40 patients infected with bacteria in our study, 13 (31%) were diagnosed with bronchitis, and 14 (33%) with nasopharyngitis. As a result, identification of inappropriate antibiotic use based on presumed viral respiratory diagnoses may not be correct. Moreover, doctors may over-diagnose some conditions such as pneumonia, acute otitis media, or acute sinusitis which are indicated for antibiotic prescription (but were not included in our study) to justify their antibiotic prescriptions. This could result in an underestimation of inappropriate antibiotic use. Table 7-1 shows a number of studies which determined inappropriateness of antibiotic use in children with ARI on the basis of presumed viral respiratory diagnoses according to evidence-based guidelines or textbooks. As can be seen from this table, rates of inappropriate antibiotic use varied from study to study and were much lower than that in our study. The reason for these differences could be explained by the disparity regarding the definitions of inappropriateness of antibiotic use. Another reason may be due to differences in study populations: unlike other studies on ARI patients, our study was carried out in respiratory examination rooms and did not include patients with otitis or sinusitis who are usually seen in the dedicated ear-nose-throat examination rooms.

Table 7-1 Studies on inappropriateness of antibiotic use in children with ARI

Authors, Year, Country	Definition of inappropriateness of antibiotic use	Study method - Participants	Study settings	Study period	Rate of inappropriate antibiotic use
Hersh et al, 2011, USA [310]	Antibiotic prescriptions for: nasopharyngitis, bronchitis, viral pneumonia, influenza, asthma, allergy, chronic sinusitis, chronic bronchitis	Cross-sectional survey on children under 18 years visiting ambulatory care settings	outpatient departments, emergency departments	2006 –2008	23.4%
Gaur et al, 2005, USA [312]	Presumed viral ARI including nasopharyngitis, URI, bronchitis and bronchiolitis	cross-sectional study of patients below 18 years old with ARI	Outpatient clinics	1995-2000	33.2%
da Cunha et al, 2003, Brazil [313]	According to WHO guidelines on ARI in children less than 5 years	Cross-sectional survey on children under 5 with ARI	Outpatient departments	1996	9.2%
Nash et al, 2002, USA [311]	Any antibiotic prescription for either URI or bronchitis	Paediatricians, family physicians, and generalists filling survey forms for patients under 18 years.	Office-based physician practices	1995 & 1998	33.9% of URI or bronchitis
Arnold et al; 1999; Canada [308]	Based on evidence-based guidelines	Review of visits of children with ARI to primary care paediatricians	Primary care clinics	1997	27%
Wang et al, 1999, Canada [309]	Based on evidence-based guidelines	computerised data on antibiotic prescription for ARI in preschool children	Population-based study	1995	49% of cases with URI, 65% with acute bronchitis or bronchiolitis, and 18% with common cold
Nyquist et al, 1998, USA [240]	Antibiotic prescriptions for common colds, URI, and bronchitis were considered as inappropriate	Physicians completing patient record forms for patients younger than 18 years	Office-based physician practices	1992	44% with common colds, 46% with URIs, and 75% with bronchitis

7.5 Impact of antibiotic overuse on the development of resistant bacteria in human gut microflora

By measuring resistant fractions of *Enterobacteriaceae* in the stool samples of our ARI outpatients after they were given antibiotics, we found a highly significant increase in the fraction of *Enterobacteriaceae* resistant to commonly used antibiotic classes such as penicillins and cephalosporins on day 7. Of note was the fact that we also observed a marked rise in *Enterobacteriaceae* resistant to the "never-used in outpatients" antibiotic classes like aminoglycosides or rarely used class such as quinolones through a major mechanism mediated by plasmids. After withdrawing antibiotics, we observed complete restorations in resistant fractions of bacteria in gut flora for all antibiotics tested, among which the resistant fractions of ceftazidime, amoxicillin and cotrimoxazole were restored to below the original baselines on Day 0. This may be because one-third of patients previously used antibiotics (consisting mostly of beta-lactam) within 1 month before presentation. Moreover, this figure of pre-treatment with antibiotics identified by parent interviews was much higher, at approximately 42%, when combined with the results of antibiotic determination by HPCL in the urine samples. The indiscriminate overuse of antibiotics in both community (pre-admission-42%) and medical settings (99.6%) demonstrated in our study poses a huge threat in terms of the widespread development of drug resistance among bacteria, given the undesired adverse effects of (oral) antibiotic use on the selection of resistant bacteria in human intestinal flora. The significant increase in the fraction of resistant bacteria under antibiotic pressure, in turn, may lead to the transfer of these resistant genes to other commensal or pathogenic bacteria through conjugation, transformation or transduction [178, 272-280], and to person-to-person spread through faecal-oral transmission. This was evidenced by a high carriage rate of extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* among healthy children who had not been using antibiotics in a number of previous studies [314-316]. For instance, ESBL-

positive *Enterobacteriaceae* were found in 2.9% of healthy Swedish preschool children [316]. A study in Senegal showed that 10% of healthy children with unknown antibiotic exposure in a remote area were found to be faecal carriers of ESBL-positive *E.coli* [315] while another study in Bamako, Mali determined that 100% of healthy children at an orphanage and 90% of adult caregivers who had close contact with these children carried ESBL-producing *Enterobacteriaceae* in their stool samples [314]. All these findings provided evidence that ESBL-producing *Enterobacteriaceae* have been circulating within the community, and thus even in the absence of direct antibiotic exposure, healthy people were at high risk of carrying multiresistant faecal bacteria through faecal-oral transmission route. Although the effect of antibiotics on resistant gut flora seems only mildly harmful and is only temporary (restoration of original fractions was observed after 28 days) in the individual patient, the selection, transmission and persistence of these resistant bacteria at the community level will likely occur, given the widespread antibiotic use in Vietnam clearly indicated by a number of studies in this country. Almost 100% of children with non-severe ARI were prescribed antibiotics in our study (as described in Chapter 3), and in Ba Vi, 90% of the children with mild ARI were treated with antibiotics in a cross-sectional study conducted in 2007 [317]. A multicenter point-prevalence study conducted in 36 Vietnamese hospitals also revealed a rate of 67.4% of in-patients receiving antibiotics and more or less one-third of these patients experienced inappropriate use of antibiotics [318]. In the community, Quagliarello et al recorded a very high rate (99%) of antibiotics dispensed by pharmacies in urban and rural districts of Vietnam for children with ARI [319]. Moreover, the consequences of resistance will become increasingly serious with more evidence of the long-term persistence of resistant genes in the human gut flora demonstrated by other studies [182, 184, 185, 189, 198, 199].

7.6 Conclusions, future work and recommendations

In summary, the important findings from this thesis were:

1. In almost all cases (562/563), antibiotics were prescribed in children presenting with mild ARI at an outpatient clinic in Ho Chi Minh City. Most of this antibiotic use was eventually deemed inappropriate because almost three-fourths of ARI children were infected with viruses and/or (atypical) bacteria, which were not susceptible to the prescribed antibiotics.
2. After such overuse of antibiotics, we recorded a short-term selection of bacteria in human gut flora resistant to the antibiotic class the patients received and also co-selection of resistance to other rarely used drugs.
3. With the use of multiplex PCR method, more than three-fourths (75.6%) of children with non-severe ARI were shown to be infected with one or more causative pathogens, most of which (72.5%) were viruses. In healthy children, this multiplex PCR helped to detect respiratory viruses and bacteria at a high rate (23.1%). Among these "healthy children", the detection rate of any pathogen in children with mild respiratory symptoms (41%) was double that in asymptomatic subjects (20%).
4. An HPLC assay method was established with high sensitivity and specificity to determine the presence of six beta-lactam antibiotics in urine. This method was used to determine accurate and reliable data about prior treatment with antibiotics which patients may have been given before consulting doctors.

Recommendations and future work

Our study underscores the need for a comprehensive programme to improve rational antibiotic use in Vietnam. We propose the following recommendations and future work in hopes of minimizing the indiscriminate use of antibiotics in ARI in Vietnam:

1. The systematic over-prescription and over-use of antibiotics in children with ARI was clearly demonstrated in this study, but the effects of this on the community in terms of direct costs of antibiotics, side effects and the introduction and persistence of resistant bacteria that may cause disease and require (more expensive) treatment remain unanswered for Vietnam. In the United States, Gonzales et al estimated that the total cost of inappropriate antibiotic use for ARI in this country was about $726 million in 1998 [320]. With respect to costs of antibiotic resistance, Roberts et al determined in a study in a Chicago teaching hospital in the US that the medical costs for a patient who acquired antibiotic-resistant infections ranged from almost $19,000 to around $29,000 while the duration of hospital stays for patients with antibiotic-resistant infections was prolonged by 6.4 to 12.7 days. The death rate among patients with antibiotic-resistant infections was 2-fold higher than that in patients with antibiotic-susceptible infections. The societal costs for patients with antibiotic-resistant infections were $10.7-15.0 million [141]. In comparison to people infected with antibiotic- susceptible organisms, patients who acquired antibiotic-resistant bacteria had much higher costs (around 1.5-fold greater), lengthier of hospital stays (more or less 1.5-fold longer) and higher mortality [143]. Schwaber and colleagues compared the outcomes of patients with bacteraemia caused by ESBL-producing bacteria and those with bacteraemia due to non ESBL producers and found longer hospital stays (1.57-fold longer), greater total costs (1.57-times more) as well as significantly higher mortality among patients infected with ESBL producers [144]. In Vietnam, all of these costs need to be addressed in future studies to elucidate the adverse impact of antibiotic overuse with respect to its cost to society. Data on the economic burden resulting from antibiotic overuse and resistance can provide a well-founded argument for strong measures against inappropriate use of antibiotics in Vietnam.

2. One of the main reasons for the high rate of outpatient antibiotic use is the difficulty in discriminating viral from bacterial infection or assessing the risk for secondary bacterial infection using readily available clues such as clinical presentation and chest X-rays. More specific (microbiologic) diagnostics usually take too long and, therefore, physicians choose to treat all possible treatable diagnoses, including a bacterial aetiology, while waiting for results. Hence, the use of these rapid diagnostic tests should be evaluated and applied in order to quickly assess the presence of viral (or bacterial) pathogens in the respiratory tract and assist treating physicians in prudent decision-making to help lower antibiotic prescription rates. The commonest aetiologies found in our study such as hRV and EV could be targets for establishing new appropriate rapid diagnostic tests. In addition, a quick point-of–care white blood cell (WBC) count, which was shown to be a useful test in aiding judicious antibiotic prescribing in ARI [321, 322], could be used, particularly in patients with symptoms which look "more severe" such as high fever. Only patients with WBC count of 15,000/mm3 or greater should be candidates for antibiotic indications. A point of care test of CRP, which was found to have predictive value for pneumonia and helped to reduce inappropriate antibiotic use in previous studies [153, 154, 323, 324], could also be employed in outpatient settings as an additional diagnostic test to predict pneumonia in patients with high concentrations of CRP.
3. Training in rational antibiotic use should be given not only to people in the community but also to medical staff including physicians as well as pharmacists (for as long as over-the-counter availability is common). Intensive education about the appropriate use of antibiotics is highly recommended for the Vietnamese public. A community-based survey in Ba Vi, Vietnam showed that almost 80% of people buy antibiotics at pharmacies without prescriptions [95]. Previous studies have shown the effectiveness

of patient education in improving appropriate antibiotic use in children [146-152]. The 2003 campaign "Get smart: Know when antibiotics work" launched by Centres for Disease Control and Prevention (CDC), which educated the public, patients and primary healthcare providers about appropriate use of antibiotics, has proved to be effective in decreasing the rate of irrational use of antibiotics in common cold by 19% and in pharyngitis by 24% [325]. However, Wheeler et al stated in their study that although parent education was effective at changing parent attitudes toward the use of antibiotics, these improvements were not associated with a corresponding improvement in prescribing rates of antibiotics in children [150]. This pointed out the crucial role of prescribers and medical staff in being aware of the appropriate indications for antibiotics. Knowledge of antibiotic resistance and the rational use of antibiotics should be provided intensively not only to students at medical and pharmacy schools, but also as part of the continuing medical education (CME) for graduated doctors and pharmacists. Razon and colleagues conducted a study to assess the impact of educational interventions for practising paediatricians on judicious antibiotic prescription for children with upper respiratory diseases and found that the use of antibiotics for URI decreased significantly after such interventions [326]. Training about the use of CRP testing and enhanced physician-patient communication for clinicians also demonstrated positive effects on appropriate use of antibiotics in patients with ARI [153, 154].

4. At government level, existing legislation on prescription-only drugs should be enforced to assure that antibiotics cannot be purchased without a doctor's consultation. It is also pivotal to formulate standard (national) therapeutic guidelines at outpatient and in-patient settings that can be implemented in every healthcare centre. Regulations and rules should be promulgated to assure that medical staff adhere strictly to these

guidelines. In addition, measures to relieve patient overload in referral hospitals should be urgently taken in order to leave physicians enough time to determine the appropriate indications for antibiotic use or to enhance clinician-patient communication, which has been shown to result in a decreased irrational antibiotic prescribing for ARI [154].

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Appendix A

Protocol of 03AV study

Outpatient Antibiotic Use in Acute Respiratory Infections (ARI) in Children's Hospital 1, Vietnam

A prospective descriptive study

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1. Background

Acute respiratory infections (ARIs) are among the most common diseases in children both in developed and developing countries. The estimated prevalence of ARIs in pediatric outpatients is about 40-60% according to Pan American Health Organization/WHO data. In Finland, a survey at 30 health centers revealed that 74% of outpatient visits were because of an ARI. Viruses are recognized as the predominant etiologic agents, causing about 60% of ARIs. In contrast, treatment of ARIs usually consists of antibiotics directed at bacterial pathogens.

A recent cross-sectional study in Outpatient Department (OPD) in Children's Hospital N1, Viet Nam showed that approximately 85% outpatients with ARI were prescribed antibiotics (unpublished results). One of the main reasons for this high rate of outpatient antibiotic use is that it is difficult to accurately discriminate viral from bacterial infection using readily available clues as clinical presentation and chest x-rays. More specific (microbiologic) diagnostics usually take too long and, therefore, physicians choose to treat all possible treatable diagnoses, including a bacterial etiology, while waiting for results.

Consumption of antibiotics in an outpatient setting is correlated with an increase of resistance among bacteria through selection of resistant bacteria under antibiotic pressure, and therefore restrictive use of antibiotics is recommended. The availability of rapid diagnostic tests that assess the presence of viral pathogens in the respiratory tract to assist in treatment decision making is needed and can help to lower the antibiotic prescription rate.

In this study children with acute respiratory illness in the outpatient clinic of Children's Hospital No 1 (Bệnh Viện Nhi Đồng 1) will be recruited. Antibiotic use will be recorded and the presence of causative organisms (viral and bacterial) will be determined by multiplex PCRs of respiratory swabs/aspirates. In addition, selection of resistant bacteria in stool will be monitored in stool samples collected on admission and follow-up.

The generated data will allow us to make retrospective conclusions on appropriate or inappropriate use of antibiotics, and will help in determining at which pathogen rapid diagnostic tests (Point of Care) should be aimed in order to effectively reduce the use of antibiotics by prescribing physicians.

2. Objectives

2.1. Primary objectives:

2.1.1 To quantify the inappropriate antibiotic use in outpatient acute respiratory infections in Children's Hospital 1, Vietnam.

2.1.2 To identify the most common viral and bacterial etiologies associated with inappropriate antibiotic use.

2.2 Secondary objectives:

2.2.1. To assess epidemiology, etiology, (pre) treatment, clinical features and outcomes of acute respiratory infections in outpatients

2.2.2. To assess the short-term effect of antibiotic use on the selection of resistant bacteria in the rectal swabs.

3. Methods and Materials:

3.1 Study design:

A prospective descriptive study

3.2 Duration of study:

One year

3.3 Study site:

Outpatient Department in Children Hospital N°1, Ho Chi Minh City, Vietnam.

3.4 Sample size calculation

It is expected that 10 patients will be enrolled each week. This will lead to a total sample size of approximately 500 patients in one year

3.5 Study population and participation criteria:

All children <16 years of age presenting to the Outpatient Department in Children's Hospital Nr 1 with ARI as the chief complaint are eligible for enrolment.

Inclusion criteria:

- Age < 16 years
- Diagnosis of ARI (see *Definitions*)
- Not admitted to the hospital
- Informed consent by parents or legal guardians

- Living in Ho Chi Minh City and agreeing to return for follow up visit after 1 week

Exclusion criteria:

- Having underlying illness (except asthma)
- Previous admission within 3 months (in any hospital or health centre).
- No consent given.

3.6 Enrollment:

Every week day from Monday to Thursday, we will screen patients until at least two patients (maximum 4 patients) meet the eligible criteria and are enrolled to the study. The number of patients enrolled each week should be between 10 and 12.

Schedule for enrollment:

Monday:	from 8-9am
Tuesday:	from 9-10am
Wednesday:	from 10-11am
Thursday:	from 1-2pm

3.7 Endpoints

3.7.1 Primary endpoint will be the appropriateness of antibiotic use in the studied population (see 3.8)

3.7.2 Secondary endpoints will be

- Clinical outcome at follow up (see 3.8)
- Etiology of ARI
- Percentages of resistant bacteria in rectal swabs at presentation and at follow-up
- Diversity of resistance associated genes in bacteria from rectal swabs
- Antibiotic levels in urine and blood on admission and urine on follow-up

3.8. Definitions

3.8.1 A case of ARI in this study is defined as follows:

A patient who presents with at least one of the following symptoms:

- Cough
- Sore throat
- Runny nose
- Nasal congestion

as the chief complaint lasting shorter than 5 days.

3.8.2 Antibiotic use is defined as follows:

3.8.2.1 Inappropriate use: antibiotics prescribed in case of a negative polymerase chain reaction (PCR) for bacteria AND positive PCR for virus

3.8.2.2 Appropriate use:

a. Appropriate choice of antibiotics: antibiotics prescribed in case of PCR positive for atypical bacteria* which are susceptible to the class of antibiotics used.

b. Inappropriate choice of antibiotics: antibiotics prescribed in case of PCR positive for atypical bacteria which are unsusceptible to the class of antibiotics used.

3.8.2.3. Unknown: PCR positive with typical bacteria, irrespective of virus test OR All tests negative.

* Atypical bacteria: bacteria other than *Streptococcus pneumoniae* and *Haemophilus influenzae*.

NB: Assessment of causative agents in this study is solely based on PCR results. A positive PCR for typical bacteria does not discriminate between asymptomatic colonization with these bacteria (~10% of patients) or respiratory illness caused by these bacteria. Hence this may underestimate the inappropriate use of antibiotics.

3.8.3 Outcome

- Complete resolution of symptoms: all symptoms present at first presentation have resolved.
- Incomplete resolution of symptoms: one or more symptoms that were present at first presentation are still present, but there are less symptoms and/or they are less severe.
- No resolution of symptoms (stable disease): symptoms present at presentation are still present, with similar severity as on presentation.
- Progressive disease: symptoms present at first presentations have become more severe, and/or the child experiences additional symptoms related to respiratory illness.

- Admitted to a hospital
- Death
- Lost to follow-up

4. Data collection:

A unique trial number will be assigned to each patient entering the trial and will be used to identify all laboratory specimens and the case record forms (CRF).

All patient information will be recorded on individual CRFs, designed for this study. Data to be recorded are baseline clinical information, underlying diseases, results of laboratory investigations, outcome and clinical information at follow-up. The information contained within the CRFs will be transferred to a computerised database and will thereafter be available to the study team. Data validation will be carried out according to Good Clinical Practice (GCP).

4.1 On day of presentation:

With every patient who is eligible for enrolment in the study, the physician will collect the following data and specimens:

4.1.1 Clinical data:

- Age
- Demographic data
- Medical history
- History of medication and antibiotic use.
- Illness day since onset
- Antibiotics prescribed by physician at this time

4.1.2 Specimen collection:

- 1 nasopharyngeal aspirate (NPA)
- 1 noseswab
- 1 throat swab
- 1 rectal swab
- Capillary blood (3 drops on filter paper)
- Urine sample: 10ml

4.2 On day of follow up (6-8 days after presentation):

4.2.1 Clinical data:

- Symptoms and signs
- Antibiotics used

4.2.2 Specimens:

- 1 rectal swab
- Urine sample

All clinical trial specimens will be labeled with the patient's trial number. Samples will be transferred daily to the laboratories at the HTD/OUCRU for processing. Investigation results will be issued to the investigators in a timely manner and a hard copy of the results will be retained in the laboratory for verification. Samples will be stored securely in freezers at the HTD/OUCRU.

5. Diagnostic investigations:

5.1 Virology and bacteriology: specimens taken form nasopharyngeal aspirate (NPA), nose swab and throat swab will be subjected to the PCR for the presence of respiratory viral and bacterial pathogens:

1. Multiplex viral PCR
    - Influenzavirus A and B
    - Enterovirus
    - Adenovirus
    - Metapneumovirus
    - Respiratory Syncytial Virus A and B
    - Rhinovirus A, B and C
    - Coronavirus OC43 229E NL63 HKU
    - Parechovirus
    - Parainfluenzavirus 1, 2, 3 and 4
    - Bocavirus
2. *Streptococcus pnenumoniae* PCR
3. *Haemophilus influenzae* PCR
4. *Chlamydophila pneumoniae* and *psitacci* PCR

5. *Mycoplasma pneumoniae* PCR
6. *Legionella* genus PCR
7. *Bordetella pertussis* PCR

5.2 Pharmacology: capillary finger blood and urine samples will be subjected to HPLC measurement of a selection the most frequently prescribed and sold drugs:

Most commonly used antibiotics in the Outpatient Department of Children's hospital Nr 1

N#	ATC code	Name of Antibiotic	Class
1	J01DC04	Cefaclor	Cephalosporin 2nd
2	J01CR02	Amoxicilline + Clavulanate	Penicillin
3	J01DD08	Cefixim	Cephalosporin 3rd
4	J01DC02	Cefuroxime	Cephalosporin 2nd
5	J01CA04	Amoxicillin	Penicillin
6	J01DD13	Cefpodoxime	Cephalosporin 3rd
7	J01FA01	Erythromycin	Macrolide
8	J01FA06	Roxithromycin	Macrolide
9	J01FA10	Azithromycin	Azalide
10	J01EE01	Cotrimoxazole	Folate synthesis inhibitor
11	J01DB01	Cephalexine	Cephalosporin 1st
12	J01FA09	Clarithromycine	Macrolide
13	J01FA02	Spiramycin	Macrolide
14	J01DB05	Cefadroxil	Cephalosporin 1st
15	J01CF04	Oxacillin	Penicillin
16	J01MB02	Nalidicid acid	Quinolone
17	J01MA02	Ciprofloxacine	Fluoroquinolone

Most commonly over the counter sold antibiotics in pharmacy

N#	ATC code	Name of Antibiotic	
1.	J01DB05	Cefadroxil	Cephalosporin 1st

2.	J01DB01	Cephalexine	Cephalosporin 1st
3.	J01FA09	Clarithromycine	Macrolide
4.	J01FA10	Azithromycin	Azalide
5.	J01DC04	Cefaclor	Cephalosporin 2nd
6.	J01CA04	Amoxicillin	Penicillin
7.	J01FA01	Erythromycin	Macrolide
8.	J01MA02	Ciprofloxacine	Fluoroquinolone
9.	J01FA02	Spiramycin	Macrolide
10.	J01EE01	Cotrimoxazole	Folate synthesis inhibitors
11.	J01MB02	Nalidicid acid	Quinolone
12.	J01DC02	Cefuroxime	Cephalosporin 2nd
13.	J01CR02	Amoxicilline + Clavulanate	Penicilline
14.	J01DD08	Cefixim	Cephalosporin 3rd
15.	J01DD13	Cefpodoxime	Cephalosporin 3rd
16.	J01FA06	Roxithromycin	Macrolide
17.	J01CF04	Oxacillin	Penicillin

5.3 Resistance Monitoring: rectal swab specimens taken on inclusion and follow-up will be subjected to culture to monitor selection of resistant bacteria:

1. Obtain dry rectal swab on presentation and at day 7 with visibly attached fecal material
2. Resuspend directly in fixed volume of 0.9% saline solution and store in fridge (2-8 °C)
3. Transport to OUCRU each day (after outpatient clinic closure)
4. Split for molecular and microbiological testing

Microbiological testing

1. Dilute saline solution 1:?

Test what dilution is appropriate on a badge of random fecal samples.

2. Plate 50ul of solution on CLED/MacConkey agar plates containing*
    a. No antibiotic
    b. Tetracyclin
    c. Amoxicillin
    d. Amoxicillin + clavulanate

e. Ceftazidime
f. Ciprofloxacin
g. Trimethoprim + Sulfamethoxazole
h. Gentamicin
i. Meropenem

*at CLSI 0.5 MIC for *Enterobacteriaceae* of each antibiotic

Store the remainder of the primary saline solution at -20C

pm: store ceftazidime and meropenem (and fluoroquinolone) resistant *Enterobacteriaceae*

3. Incubate O/N
4. Count the number of colonies on each plate or on a quadrant of each plate when there are too many. If plates are confluent, retest with 1:10 diluted sample
5. Endpoint is number of colonies on b (N_b) divided by the number of colonies on a (N_a), likewise N_c/N_a, N_d/N_a, N_e/N_a, N_f/N_a, N_g/N_a, N_h/N_a and N_i/N_a. Fractions of Day 0 samples will be compared with fractions of Day 7 samples

5.4 Detection of resistance associated genes.

Resistance associated genes in *Enterobacteriaceae* will be detected using specific PCRs aimed at *qnr* genes or integrons.

6. Data management and analysis:

All clinical data, samples collection forms and questionnaires will be stored in a research database, and analyzed using SPSS or STATA software.

7. Consent procedures

Written informed consent will be obtained from parents/legal guardians of all patients participating in the study. All participants and their parents/legal guardians will be informed about the study by a letter, translated in Vietnamese. For patients or their parents/legal guardians who are illiterate, the study doctor will explain the study verbally. Consent will be obtained with a fingerprint.

For unconscious patients, consent will be obtained from their legal guardians. In case an unconscious patient is not accompanied by their legal guardians, the patient will not be approached to discuss the study until the patient is stable.

8. Ethical Considerations

The study will be reviewed by the Scientific and Ethical Committee of the Children's Hospital N1 and the Oxford University Tropical Research Ethical Committee (OXTREC).

Appendix B

Case Report Form – 03AV study

Outpatient Antibiotic Use in Acute Respiratory Infections (ARI) in Children's Hospital 1, Vietnam

Enrollment		**Enrol**
Site Number	Patient Number	Enrollment date
[_|_]	[0|2|A|V|_|_|_]	[_|_]/[_|_]/[_|_|_|_] dd mm yyyy

1. GENERAL INFORMATION

a. Patient Initials: [_\|_\|_\|_\|_\|_]

b. Date of Birth (dd/mm/yyyy): [_\|_]/[_\|_]/[_\|_\|_\|_]

c. Sex: O Male O Female

d. Address (District):_____

e. Date of visit (dd/mm/yyyy): [_\|_]/[_\|_]/[_\|_\|_\|_]

f. Reason of visit: _____

2. INCLUSION CRITERIA

a. Illness < 5 days O Yes O No O Unknown

Day of illness: [_\|_] days

b. Informed consent obtained: O Yes O No O Unknown

c. Living in Ho Chi Minh O Yes O No O Unknown

d. Agree to return for follow up after 1 week O Yes O No O Unknown

e. At least one of the following symptoms:

- Cough O Yes O No O Unknown

  If yes, productive O Yes O No O Unknown
- Sore throat O Yes O No O Unknown
- Runny nose O Yes O No O Unknown
- Nasal congestion O Yes O No O Unknown

f. Does the patient meet the inclusion criteria? O Yes O No

3. EXCLUSION CRITERIA

a. Underlying illness other than asthma: O Yes O No O Unknown

If yes, which illness:_____

b. Previous admission within 3 months (in any hospital or health center):

O Yes O No O Unknown

c. Does the patient meet the exclusion criteria? O Yes O No

4. EPIDEMIOLOGICAL AND MEDICAL HISTORY

a. Number of persons in household (including adults and children):

- Total [__|__]
- < 5 years old [__]
- 5-15 years old [__]
- 15-20 years old [__]
- > 20 years old [__]

b. Number of rooms in house/apartment [__]

c. Previous hospital admissions for respiratory problems?

O Yes O No O Unknown

- If yes: how many times? O < 3 O 3-10 O >10
- Most recent admission O< 1 month O 1-12 m O > 12m
- Reason (if known)? _____

d. Previous hospital admissions for cardiac problems?

O Yes O No O Unknown

- If yes: reason (if known)? _____

e. Previous hospital admissions for other reasons?

O Yes O No O Unknown

- If yes, reason (if known)? _____

f. Were other household members ill during past three weeks?

O Yes O No O Unknown

- If yes, details: _____

g. Were any classmates or playfriends ill during past three weeks?

O Yes O No O Unknown

PRESENTATION		Present
Site Number	Patient Number	Presentation date
[_|_]	[0|2|A|V|_|_|_|_]	[_|_]/[_|_]/[_|_|_|_] dd mm yyyy

1. HISTORY ON PRESENTATION

a. Other symptoms:

- Conjunctivitis: O Yes O No O Unknown
- Dyspnea O Yes O No O Unknown
- Cyanosis O Yes O No O Unknown
- Diarrhoea O Yes O No O Unknown
- Rash O Yes O No O Unknown

If yes, specify: _____

b. Does the child have a history of asthma? O Yes O No O Unknown

c. Does the child use antibiotics now? O Yes O No O Unknown

If yes, PLEASE COMPLETE FORM ANTIBIOTICS

d. Are these antibiotics used for the current chief complaint?

O Yes O No O Unknown

- If No, please specify: _____

e. Has the child used antibiotics in the last month? O Yes O No O Unknown

- If yes, PLEASE COMPLETE FORM ANTIBIOTICS

2. EXAMINATION ON PRESENTATION

Vital signs:

- Pulse: [_|_|_] / minute
- Temperature: [_|_].[_]°C. Fever: O Yes O No

Cardiovascular:

- Heart sounds O Normal O Abnormal
  - o Describe: .....

Respiratory:

- Breath rate: [_|_|_] / minute.
- Chest indrawing O Yes O No
- Stridor O Yes O No

- Rhonchi/Wheezes O Yes O No
- Crackles O Yes O No
- Added sounds: Left: O Yes O No
    - Describe: .....
      Right: O Yes O No
    - Describe: .....

Abdomen: O Normal O Abnormal

- Describe: .....

CNS: O Normal O Abnormal

- Describe: .....

3. DIAGNOSIS ON PRESENTATION: _____

4. TREATMENT

a. Antibiotics prescribed this visit? O Yes O No

- If yes, PLEASE COMPLETE FORM ANTIBIOTICS

b. Other treatment prescribed: O Yes O No

If yes, please specify: _____

5. SPECIMEN COLLECTION ON PRESENTATION

Nasopharyngeal aspirate:	O Yes	O No
Throat swab	O Yes	O No
Nose swab	O Yes	O No
Blood	O Yes	O No
Urine	O Yes	O No
Rectal swab	O Yes	O No

FOLLOW UP 1 WEEK		FU
Site Number	Patient Number	Follow up date
[_|_]	[0|2|A|V|_|_|_]	[_|_]/[_|_]/[_|_|_|_] dd mm yyyy

1. FINDINGS ON FOLLOW -UP AFTER 1 WEEK

a. Days after presentation: [_|_] days

b. Number of pills / Volume left of antibiotics prescribed on first visit: [_|_]

c. Use of other than antibiotics prescribed on first visit during last week?

O Yes O No OUnknown

If yes, PLEASE COMPLETE FORM ANTIBIOTICS

2. HISTORY ON FOLLOW-UP

a. Symptoms:

• Cough :	O Yes	O No	OUnknown
o if yes, productive	O Yes	O No	OUnknown
• Sore throat	O Yes	O No	OUnknown
• Runny nose	O Yes	O No	OUnknown
• Nasal congestion	O Yes	O No	OUnknown
• Conjunctivitis	O Yes	O No	OUnknown
• Dyspnea	O Yes	O No	OUnknown
• Cyanosis	O Yes	O No	OUnknown
• Diarrhoea	O Yes	O No	OUnknown
• Rash	O Yes	O No	OUnknown

o if yes, specify: _____

3. EXAMINATION ON FOLLOW-UP

Vital signs:

- Pulse: [_|_|_] / minute
- Temperature: [_|_].[_]°C. Fever: O Yes O No

Cardiovascular:

- Heart sounds O Normal O Abnormal
  - o Describe: .....

Respiratory:

- Breathe rate: [__|__|__] / minute.
- Chest indrawing O Yes O No
- Stridor O Yes O No
- Rhonchi/Wheezes O Yes O No
- Crackles O Yes O No
- Added sounds: Left: O Yes O No Describe: _____
  Right: O Yes O No Describe: _____

Abdomen: O Normal O Abnormal

  - Describe: _____

CNS: O Normal O Abnormal

  - Describe: _____

Final diagnosis: _____

4. SPECIMEN COLLECTION ON FOLLOW-UP

Urine: O Yes O No

Rectal swab: O Yes O No

5. OUTCOME: [___]

(1) Completely recovery
(2) Partial recovery
(3) Unchanged
(4) Worsening
(5) Admitted to hospital
(6) Death
(7) Lost to follow-up
Reason: _____

1. FINDINGS ON FOLLOW -UP AFTER 1 MONTH

a. Days after presentation: [__|__] days

c. Use of antibiotics after follow-up visit after 1 week?

O Yes O No OUnknown

If yes, PLEASE COMPLETE FORM ANTIBIOTICS

2. HISTORY ON FOLLOW-UP

a. Symptoms:

- Cough : O Yes O No OUnknown
  - o if yes, productive O Yes O No OUnknown
- Sore throat O Yes O No OUnknown
- Runny nose O Yes O No OUnknown
- Nasal congestion O Yes O No OUnknown
- Conjunctivitis O Yes O No OUnknown
- Dyspnea O Yes O No OUnknown
- Cyanosis O Yes O No OUnknown
- Diarrhoea O Yes O No OUnknown
- Rash O Yes O No OUnknown
  - o if yes, specify:

_____

4. SPECIMEN COLLECTION ON FOLLOW-UP

Rectal swab: O Yes O No

5. OUTCOME: [___]

(1) Complete recovery from first disease

(2) Incomplete recovery

(3) New diseases requiring antibiotics

- if yes, specify:

_____

ANTIBIOTICS **AB**

Site Number	Patient Number	Follow up date
[__|__]	[0|2|A|V|__|__|__]	[__|__]/[__|__]/[__|__|__|__] dd mm yyyy

Name	Dose	Start Date *dd/mm/yy*	Stop Date *dd/mm/yy*	Comments
		__/__/__	__/__/__	
		__/__/__	__/__/__	
		__/__/__	__/__/__	
		__/__/__	__/__/__	
		__/__/__	__/__/__	
		__/__/__	__/__/__	
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		__/__/__	__/__/__	
		__/__/__	__/__/__	
		__/__/__	__/__/__	
		__/__/__	__/__/__	

Appendix C

Protocol of 01RS study

Screening for the presence of respiratory viruses in specimens of healthy children

1. Background

Acute respiratory infections (ARIs) are among the most common diseases in children both in developed and developing countries. The estimated prevalence of ARIs in pediatric outpatients is about 40-60% according to Pan American Health Organization/WHO data. In Finland, a survey at 30 health centers revealed that 74% of outpatient visits were because of an ARI. Viruses are recognized as the predominant etiologic agents, causing about 60% of ARIs.

But a study in the Gambia showed that respiratory viruses can also be cultured from a considerable percentage of healthy children. Although the association with disease for several respiratory viruses has been well established (Influenza viruses, RSV), asymptomatic carriage is also a well known occurrence. For other viruses the association with diseases is less well defined, and the significance of detection in these viruses in children with respiratory illness is unknown.

Because we are currently performing multiple studies on respiratory infections in either out- or inpatient children in Children's Hospital 1 and 2, involving diagnostics by multiplex PCR on 15 respiratory viruses, we intend to set up a control group of healthy children to be able to compare incidence rates and copy numbers of respiratory viruses in children with and without disease and assess the significance of detection of these viruses.

In addition, these samples will be used as a healthy control cohort to compare the expression profiles with those of hospitalized children with RSV infection.

2. General aim of the study

To take respiratory samples from healthy children to assess the presence of respiratory viruses and to establish a control group for ongoing work on respiratory infections in- and outpatients in Children's Hospital 1 in Ho Chi Minh City.

3. Study site(s)

- The vaccination outpatient clinic in Children's Hospital 1 in Ho Chi Minh City

4. Inclusion criteria:

- Age < 16 years
- Informed consent by parents or legal guardians

5. Exclusion criteria:

- Having fever
- Having underlying illness (except asthma)
- Previous admission within 3 months (in any hospital or health centre).
- No consent given.

6. Methods

We will sample 240 children once during the course of 1 year.

- Samples will be taken every month on the vaccination outpatient clinic. Each month we will sample approximately 5 children <1 year of age, 10 children between 1 and 4 years of age, and 5 children of 5 years of age or older.

We will take a swab from the nasal cavity and the throat of the children, which will be put in RNA later after collection and subsequently stored at 4 °C and transported to OUCRU.

Samples will be analyzed for the presence of respiratory viruses using an established multiplex PCR, and for expression profiles using a microarray platform at the Genome Institute in Singapore.

7. Consent procedures

Children's parents or legal guardians will be given a patient information sheet and will be asked to consent at the outpatient clinic. Only children whose parents or legal guardians have completed the consent form will be included in the study.

8. Ethical considerations

A unique study number will be assigned to each child entering the study and this will be used to identify the laboratory specimen. All patient information will be recorded on individual log sheets, designed for this study. Data to be recorded are sex and date of birth. The information contained within the log sheets will be transferred to a computerized database and will thereafter be available to the study team.

The study will be reviewed by the OXTREC.

Appendix D

Case Report Form – 01RS study

Screening for the presence of respiratory viruses in specimens of healthy children

Label:

Date(dd/mm/yy): [__|__]/[__|__]/[__|__]

Initials: [__|__|__|__|__|__] Sex (circle): Male / Female

Date of birth (dd/mm/yyyy): [__|__]/[__|__]/[__|__|__|__]

Inclusion criteria:

1. Age < 16 years ☐
2. Informed consent by parents or legal guardians ☐

Exclusion criteria:

3. Having fever ☐
4. Having underlying illness (except asthma) ☐
5. Previous admission within 3 months (in any hospital or health centre) ☐
6. No consent given ☐

Questionnaire

7. Currently, does the child have any respiratory symptoms? O Yes O No
   If yes, please specify: [_____]
8. Did the child visit a doctor for respiratory symptoms within the last 2 weeks? O Yes O No O Unknown
9. Is the child currently using antibiotics for respiratory infection? O Yes O No O Unknown

Study staff signature _____ **Date:** _____

Appendix E

OxTREC's and Children's Hospital 1's Approvals

Oxford Tropical Research Ethics Committee
OXTREC
University of Oxford
Room 8, Ground Floor, Manor House
The John Radcliffe, Headington, Oxford OX3 9DZ
tel. +44 (0) 1865 743005, fax +44 (0) 1865 743 002
e-mail: Fiona.Maclean@admin.ox.ac.uk

28th July 2008

Elaine Stockwell
Head of Clinical Trials Unit
Hospital for Tropical Diseases
190 Ben Ham Tu, Quan 5
Ho Chi Minh City
Viet Nam

Dear Elaine

Full Title of Study: Outpatient Antibiotic Overuse in Acute Respiratory Infections (ARI) in Children's Hospital No.1 Vietnam.

OXTREC Reference Number: 31 08

Thank you for your letter dated 28th July 2008. Clarifying the use of rectal swabs as requested by the OXTREC committee.

I am happy as to be able to take Chairman's action and give approval for the study.

We look forward to receiving your annual report with interest.

Yours sincerely

Dick.

Richard Mayon-White
OXTREC Chair

Cc Dr Van Doorn Hospital for Tropical Diseases – Ho Chi Minh City

OTHER PUBLICATIONS

1. South East Asia Infectious Disease Clinical Research Network. ***Effect of double dose oseltamivir on clinical and virological outcomes in children and adults admitted to hospital with severe influenza: double blind randomised controlled trial***. BMJ. 2013; 346:f3039. doi: 10.1136/bmj.f3039.

2. van Doorn, H.R., N.V. Kinh, H.M. Tuan, T.A. Tuan, **N.N.Q. Minh**, J.E. Bryant, V.T.T. Hang, L.T.T Uyen, L.Q. Thinh, T.T.N. Anh, N.P.H. Lan, N.V. Trung, W. Taylor, L. Merson, H.F.L. Wertheim, J. Farrar, M. Wolbers, N.V.V. Chau and M.D. de Jong. ***Clinical Validation of a Point-of-Care multiplexed in vitro immunoassay using monoclonal antibodies (MSD® Influenza Test) in 4 hospitals in Vietnam***. J Clin Microbiol. 2012; 50(5):1621-5. doi:10.1128/JCM.00085-12